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# Traits associated with chilling temperatures in maize.

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# TRAITS ASSOCIATED WITH CHILLING TEMPERATURES IN MAIZE

A Dissertation Presented

By

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Abstract

Traits Associated with Chilling Temperatures in Maize

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Four maize inbreds, A502, B73, Mp305, and (V3xB14)-2-1, some of the F1 hybrids from these inbreds, and the species Zea diploperennis were subjected to several chilling treatments in order to observe genotypic differences and to investigate the genetic relationships between several types of responses to low temperature.

When seeds of 6% moisture imbibed water at 2C, there were strong genotypic differences. Survival of the inbred Mp305 was only about 1% while survival of some of the hybrids was 30-90%, similar to non-chilled seeds.

The growth rates of A502 and Mp305 at 16C were much slower than for the inbreds B73 and (V3xB14)-2-1. (V3xB14)-2-1 was the fastest growing inbred at 16C; however it was also the most chlorotic inbred at this temperature. In an F2 generation of (V3xB14)-2-1xA502, the correlation between chlorophyll content and growth at 16C was .136 (not significant). The growth rate of Zea diploperennis was reduced as much

at 16C as were growth rates of several of the maize genotypes. On the other hand chlorophyll content of Zea diploperennis was as high for plants grown at 12C as for those grown at 22C, in sharp contrast to all maize genotypes which were quite chlorotic when grown at 12C.

When plants grown at 22C were exposed to a chilling treatment of 2-3C and then allowed to grow an additional week at 22C, all genotypes developed necrotic tissue on the leaves. There were large genotypic differences in the relative effects of this chilling treatment. Neither a water-saturated atmosphere nor a warmer root zone prevented damage to leaves. Zea diploperennis was as affected by this chilling treatment as were several of the maize genotypes. The amount of damage to plants by this chilling treatment was found to be partly dependent upon size of plant at the time of chilling.

There were no differences in respiration and fatty acid content of maize genotypes grown at 22C. Respiration of Zea diploperennis was lower at 16C and 22C than for maize, but was similar at 10C. When plants were grown at 16C, respiration of the maize inbreds A502 and Mp305 was markedly reduced at 10C, 16C, or 22C, whereas respiration of some hybrids was not affected by the lower growth temperature.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

# C H A P T E R  I

# LITERATURE REVIEW

## Introduction

"Chilling injury" is a term used to describe a variety of symptons which develop in plants as a result of exposure to temperatures above freezing and below about 10-12C (68). Chilling temperatures can affect plants during imbibition, germination, seedling growth, fruit set, and during post harvest storage. The primary focus of this review will be the affects of chilling temperatures during seed imbibition, germination, and seedling growth.

It is common knowledge that warm season crops such as soybeans, tomatoes, peppers, cotton, squash, melons, and lima beans cannot be planted in temperate climates until warm weather arrives. However, there are surprisingly few research studies which have measured the affects of chilling temperatures on yields. A few investigators have observed reductions in yields of cotton that was chilled during imbibition (22, 137). Poor stands and poor yields of some maize inored lines were noted when seeds were planted in early spring (38).

It is a reasonable assumption that poor germination and slow seedling growth of chilling sensitive cultivars can reduce productivity. There is abundant evidence that low temperature can reduce germination, emergence, or seedling growth in crop plants. Affected plants include

cotton (13, 17, 19, 67, 137) maize (12, 14, 15, 23, 36, 37, 45, 83, 86, 89), Phaseolus vulgaris (34, 60), lima beans (111, 113, 138), soybeans (11, 96) and tomatoes (57).

## Physiological Effects of Chilling Temperatures

In addition to poor germination and growth, a number of other effects have been observed when sensitive plants have been exposed to low temperatures. Some of the commonly observed responses include reduced germination following cold imbibition, solute leakage from chilled tissues, interference with membrane potential, uptake and transport anomolies, organelle abnormalities, reduced photosynthesis, reduced chlorophyll content, respiratory anomolies, and non-linear Arrhenius plots of enzyme activities and other processes. This section is an enumeration of the many types of physiological responses of plants that have been chilled. A knowledge of the range of physiological responses is essential for the development of theories regarding the mechanisms of chilling injury. Proposed mechanisms of chilling injury will be presented in the next section, Mechanisms of Chilling Injury.

Several studies have demonstrated that the initial imbibition of water by the seed is a very chilling sensitive stage in many plants, especially when moisture content is low prior to imbibition. Cotton seeds that imbibed water at 5C produced seedlings with root abnormalities, but these abnormalities were prevented when seeds were exposed to one or more hours of water at 30C prior to imbibition (19).

Two periods of sensitivity to cold imbibition occurred in cotton, one during initial imbibition and the other 18-32 hours later (13).

Lima bean seeds are especially sensitive to cold imbibition, showing increased solute leakage and reduced survival after imbibition at 5C (111), and reduced seedling vigor following imbibition at 15C (113). As in cotton, prior imbibition of warm water had a protective effect.

Maize seeds are also sensitive to imbibitional chilling. Imbibition at 5C by low moisture seeds resulted in aborted radicals, proliferation of seminal roots, delayed growth, reduced survival (14), and stelar lesions (23). As with cotton and lima bean, higher moisture content in seeds prevented or reduced the adverse effects of imbibitional chilling. Similar results have been observed with soybean. Imbibition at 5C by low moisture seeds resulted in reduced survival and growth, and decreased ability to use cotyledonary reserves (96). Higher moisture content protected seeds (61, 96).

Several studies have shown increased solute leakage from tissues following or during chilling. Increased solute leakage from soybeans was observed during exposure to temperatures below 10C (11). Increased solute leakage from leaves has been observed following chilling of maize (26), cucumber (148), and Passiflora species (100). Increased solute leakage occurred from cotton radicals following chilling (20). However, Murata and Tatsumi (92) found that increased solute leakage following chilling was not necessarily a general property of chilling sensitive plant tissues. Increased solute leakage following chilling was observed for cucumber, pickling melon, and snap bean, but not for bell pepper and eggplant fruits.

Another response to chilling temperatures involved the re-establishment of membrane potential following chilling. Chilling resistent oats as well as chilling sensitive maize showed membrane depolarizations when temperature was lowered. However, repolarization occurred in the light for oats but not for maize (56).

A number of investigators have observed respiratory anamolies during or after chilling. A three fold increase in respiration of leaves of Episcia reptans was seen after chilling at 5C for 80 minutes, and the respiratory burst coincided with onset of visible injury. During the increased oxygen consumption there was not a corresponding increase in carbon dioxide production, suggesting peroxidation of lipids (143). A respiratory increase was noted in maize following chilling at 0.3C for 24 to 48 hours. The increase was at least partially due to uncoupling of oxidative phosphorylation (26). Increased oxygen uptake 2 to 3 hours after chilling at 2C for 48 hours occurred in cucumber hypocotyls. Increased solute leakage was observed immediately after chilling, suggesting that the respiratory burst may have been a secondary effect (119). The respiratory burst of cucumber hypocotyls during chilling was seen to involve a switch towards greater cyanide-insensitive respiration and away from the normal cytochrome pathway (59). When cotton was chilled for 12 hours at 2.8C, increased respiration was apparent upon rewarming to 25C. Longer chilling periods reduced the capacity of leaves and roots to re-establish normal respiration (3). Chilling of cotton seedlings lowered ATP content (132).

Uptake and transport processes may also be affected by chilling. Leucine uptake by tomato leaf fragments was more reduced in chilling sensitive genotypes (102). There was no measurable nitrate absorption by the chilling resistent barley plant at 13C (144). Perhaps absorption is adversely affected at even higher temperatures in chilling sensitive plants. In Digitaria decumbens a night temperature of 10C prevented accumulations of starch in the chloroplasts from being translocated out of the chloroplasts, and photosynthetic rates were reduced (49).

Structural abnormalities have been noted in chilled cells. Tomato seedlings chilled at 5C showed loss of turgor, reduced vacuolization, reductions in cytoplasm, reductions in vacuolar protein bodies, deposits of substances in cell walls, and disorganization of organelles (54). Similarly, chilled tomato cotyledons showed membrane deteriorations. Tonoplasts were affected first followed by chloroplast membranes, mitochondrial membranes, the endoplasmic reticulum, peroxisome membranes, nuclear membranes, and finally the plasmalemma (53). In chilling sensitive species of Passiflora there was a rapid deterioration chloroplasts following chilling at 0C (125). Mitochondria from chilling resistent plants had a greater capacity for swelling in hypotonic solution than did mitochondria from sensitive plants (72). In maize, large callose deposits were found around the shoot apex and the root cap at 20C but not at 26C (128). Wilting occurred in chilled cucumber seedlings even when roots were kept warm (63).

An increase in the concentration of free nucleosides in cotton seedlings was noted following chilling (133). This suggests the

possibilities that nucleic acids were either being broken down or that sequencing of nucleosides into nucleic acids was being inhibited more than was synthesis of nucleosides. If the synthesis of nucleic acids is affected by low temperatures, one would also expect protein synthesis to be affected. In another study (64) ribonuclease activity and polysome formation were found to be reduced in chilled cotton cotyledons, which is an indication that protein synthesis was probably also reduced.

Isocitratase activity in cotton seeds was reduced by chilling (95, 127). Effects of chilling *in vitro* on several other enzymes are presented in the next section, Mechanisms of Chilling Injury.

Protoplasmic streaming was reduced or eliminated by chilling at 10C in several chilling sensitive plants (65).

A number of studies have shown effects of chilling on photosynthesis or the photosynthetic apparatus. Light-saturated photosyntic rates and density of mesophyll photosynthetic units of maize leaves declined with increasing duration of low temperature treatments (136). Proton and PMS-phosphorylation of cucumber chloroplasts were reduced by a treatment of 4C in the light; recovery was slower in the light than in the dark (42). The effects of chilling on photosynthesis were more severe in the light than in the dark (43). Resistant species of *Passiflora* showed no change in photoreduction following chilling at 0C but photoreduction was decreased in sensitive species (125). Photosynthetic capacities of leaves of *Sorghum* , *Maize*, and *Pennisetum* were reduced by high light coincident with low temperature (135). Photosynthetic rates of cultivars of alfalfa adapted to colder climates were less affected than

the photosynthetic rates of cultivars adapted to warmer climates (105). However, Hill activity was found not to be closely related to chilling sensitivity in several Passiflora species (27).

Chilling has been shown to have effects on chlorophyll content in sensitive plants, especially when high light intensity was coincident with low temperature. Etiolated maize seedlings synthesized very little chlorophyll at 16C and 3000 foot candles, but at a higher temperature or at a lower light intensity more chlorophyll was synthesized. Plants allowed to green first did not become chlorotic (81). Chlorophyll of maize plants declined with increasing duration of low temperature (136). High light and low temperature reduced chlorophyll in soybean leaves more than did low light coincident with low temperature (9). High light intensity and low temperature inhibited chlorophyll development in Sorghum but not in barley (80). When the maize mutant MII was grown at 15C plastids were deficient in ribosomes and the ultrastructure of the plastids was abnormal. The mutant contained essentially no chlorophyll below 17C (84).

Chlorotic banding of chilled leaves was first reported by Faris (1926). Slack et al. (1974) investigated the nature and possible causes of "Faris banding." Temperatures of -3C to 4C produced banding in three tropical species, Digitaria smutsii, Paspalum dilatum, and several Sorghums. A single band appeared about 48 hours after the chilling treatment was terminated and persisted until leaf senescence. There was a deficiency of chlorophyll in mesophyll plastids but all parenchyma sheath cells contained green plastids. Chlorotic plastids contained fewer ribosomes.

When the logarithm of various cellular processes is plotted against 1/K (an Arrhenius plot), chilling sensitive species have sometimes produced non-linear slopes with changes in slope occurring at chilling temperatures. Arrhenius plots of respiration of mitochondria from the chilling sensitive plants, sweet potato, tomato, and cucumber, produced non-linear slopes with changes in in slope at about 12C (70). No changes in slope were observed for several chilling resistent plants, beet, potato, and cauliflower. There were two changes in slope in Arrhenius plots of succinate oxidase activity of chilled sweet potato, one at 10C and the other at 17 C (150). There were breaks in Arrhenius plots of electron transport and phosphorylation in a blue-green alga. The temperatures at which the breaks occurred were dependent on the temperature during growth (90). An Arrhenius plot for the amount of exudate from cut soybean stems showed a break at 8.7C when plants were grown at 17/11C (day/night) and 14C when plants were grown at 28/23C. There were no breaks in Arrhenius plots for broccoli (75).

In summary a number of symptoms have been noted during or following chilling of sensitive plants. Some frequently observed symptoms include reduced survival and growth following cold imbibition, organelle abnormalities, electrolyte leakage, abnormal chlorophyll development, respiratory anomalies, and reduced photosynthesis. Arrhenius plots of mitochondrial respiration, enzyme activities, and other processes have shown changes in slope at chilling temperatures for sensitive species. The wide range of symptoms suggests that chilling injury encompasses more than one phenomenon. Responses may be classified into 3

groups: First, there may be structural damage to tissues or molecules. Evidence of this in includes the observation that some chilled membranes become leaky and that upon chilling, organelles of some plants appear to deteriorate. Second, some cellular processes might be slowed more than others so that imbalances occur. There is little evidence to support the existence of biochemical imbalances. However, one type of imbalance was observed. In some plants chlorophyll synthesis is severely inhibited at temperatures which still permit growth. The occurrance of low stomatal resistance along with low root permeability at at 5C might also be viewed as a type of imbalance. There may be many other types of imbalances at chilling temperatures. The third general type of response to low temperature could be thought of as an effect similar to dormancy. Below some chilling temperature plants may simply fail to germinate or plants which have already germinated may cease growing. This perhaps should not be considered "chilling injury" if the plants can resume normal growth when temperature increases. However, poor growth could reduce crop productivity, especially if growth were greatly reduced at relatively mild chilling temperatures.

Theories about the mechanisms injury must deal with the diversity of responses to chilling temperatures.

## Mechanisms of Chilling Injury

Crookston (29) found that one night of chilling at 5C severely inhibited photosynthesis in Phaseolus vulgaris the following day. RUBP

carboxylase activity was not affected, but stomatal resistance was increased following chilling. Thus it was suggested that chilling induced a water stress which caused stomates to close and photosynthesis to be reduced due to reduced carbon dioxide uptake. Wilson (1976) also proposed that a temperature-induced water stress was the major factor in chilling injury of Phaseolus vulgaris. Evidence that water stress was important included the facts that drought-hardened plants were less susceptible to chilling, that 100% relative humidity protected plants from chilling, and that stomates of non-hardened plants were open during chilling at 5C while root permeability to water was very low. However, plants that were kept at 100% relative humidity and 5C eventually were damaged. Furthermore, although water stress was undoubtedly involved, one must answer the question of why stomates were open at chilling temperatures. A search for the basic mechanisms of chilling injury must ultimately involve an investigation of events at the molecular level.

The three macromolecules in the cell which are most likely to be primary sites of chilling injury are the molecules most involved with cellular biochemistry: lipids, proteins, and nucleic acids. Theories about the mechanisms of chilling injury must deal with possible effects of low temperature on these three types of molecules.

Tait (134) proposed that low temperatures cause death or injury to sensitive plants due to the solidification of "protoplasmic lipids." Several contempory researchers (71, 114, 117) regard membrane lipids as the primary sites of chilling injury. Membranes of sensitive plants are thought to undergo phase transitions from the normal liquid-crystalline

state to the abnormal solid-gel state as the temperature is lowered into the chilling range. Once in the solid-gel state, normal membrane permeability is presumably disrupted. Altered membrane permeability could explain why electyrolyte leakage has been observed in chilled tissues. Altered membrane permeability could also explain a number of other symptoms of chilling injury. Leaky membranes might cause biochemical imbalances. Damage to membranes of germinating seeds might release organic materials which stimulate the growth of pathogens which prevent emergence. Altered membrane permeability of membranes might also account for the paradoxical opening of stomates at low temperature. It is possible that the contents of cells near the guard cells might leak out, exposing the guard cells to a higher water potential. This explanation would require that the membranes of the guard cells are still functional to some extent. An alternative explanation is that chilling induces some other type of structural change in leaves which destroys the mechanical functioning of guard cells. Gradual loss of solutes during chilling could explain why the extent of chilling injury increases with duration of low temperature. Finally, altered permeability of the tonoplast could result in release of proteolytic enzymes which could disrupt the cell (152). In fact vacuoles have been found to be the first organelles to be visibly affected (53, 54).

In addition to altering permeability, solidified membrane lipids could result in other effects. Enzyme which are strongly dependent on their membrane lipid environment for activity may become less active or inactive at chilling temperatures. Non-linear slopes in Arrhenius plots

of the activities of membrane-bound enzymes might be attributed to lipid phase changes. Solidification of lipids could also be the cause of cessation of cytokinesis and protoplasmic streaming (71, 98). The finding that chilling injury is a function of time as well as temperature (71) could be explained by the development of biochemical imbalances created because some enzymes are less closely associated with membranes and would not be as affected by lipid phase changes. The occurrance of metabolic imbalances would imply the accumulation of metabolic intermediates following prolonged chilling. There is little evidence that metabolic intermediates do, in fact, accumulate in sensitive growing plants, although accumulations of acetaldehyde and ethanol were found in chilled banana fruits (91).

Several researchers have investigated the effects of removing the lipids from membrane-bound enzymes with the hope of elucidating the role of lipids in chilling injury. If lipid phase changes are the causes of breaks in Arrhenius plots removal of the lipids from the affected enzymes should eliminate the breaks. A break at 12C in an Arrhenius plot of maize PEP carboxylase activity was eliminated when the enzyme was washed with the detergent Triton-X (107). A similar response has been reported for succinate oxidase (79). The elimination of these breaks in Arrhenius plots when lipid material is removed provides support for the idea that lipids are chilling sensitive sites.

The temperature of phase change is influenced by the degree of unsaturation of membrane lipids such that increases in the degree of unsaturation will lower the temperature of phase transition. A number

of researchers have examined the relationship between the degree of unsaturation of membrane lipids and chilling sensitivity. Unsaturation of phospholipids was greater in chilling resistent broadbeans than in chilling sensitive lima beans (35). Unsaturation of mitochondrial membranes was greater in the chilling resistent plants--peas, cauliflower, and turnip--than in chilling sensitive plants--tomatoes, sweet potatoes, and maize (72). There was a close correspondence between the degree of unsaturation of lipids in chloroplasts and the chilling sensitivity of the photosynthetic apparatus in alfalfa (105).

A number of investigators have found increases in unsaturation when plants or other organisms are transferred to lower temperatures. Sinensky (123) termed this a "homeoviscous adaptation" of the membranes. Organisms adapted to a wide temperature range are thought to be able to maintain a more or less constant state of viscosity in their membranes. Leaf lipids of Atriplex lentiformis were more saturated when plants were grown at 43/30C (day/night) than when grown at 23/18C (104). Unsaturation of phospholipids in cotton and beans increased when plants were chill-hardened at 12C for 4 days (146). There was a rapid desaturation of fatty-acids of soybean roots when temperature was decreased from 30C to 15C, and saturation increased when temperature was raised. Mitochondrial lipids changed more rapidly than lipids in the plasmalemma (118). Lower growth temperatures have been found to stimulate the synthesis of more unsaturated fatty-acids in a psychrophilic fungus (73). Unsaturation of fatty acids of chloroplast lamellae increased in white pine upon onset of winter (33). Lowering

the growth temperature of the blue-green alga Synechococcus lividus caused lipids to become more unsaturated (41). E. coli was able to maintain a constant state of viscosity of its membranes over a wide range of temperatures. The homeoviscous adaptation mechanism of E. coli involves not only the degree of unsaturation but also the chain length such that short chain lengths have an effect similar to unsaturation (123). The minimum growth temperature of Archeoplasma laidlawii was lowered by feeding it unsaturated fatty acids (78).

The presence of sterols in lipids is known to cause a flattening out of the phase change--the change occurs at the same temperature but the lipid is more fluid at the phase transition temperature. A content of 33% cholesterol completely eliminated any detectable transition (32). Presumably, cholesterol has a moderating effect on phase change of membranes as well, although one would not find levels as high as 33% in living membranes. Yeast and fungi which grow over a wide range of temperatures have large amounts of sterols in their membranes (32). Little work has been done on the effects of sterols in plant membranes in regard to cold sensitivity, even though there are significant levels of sterols in the plasmalemma. The mole fraction of sterol/phospholipids in the plasmalemma was found to be 1.0 to 1.2 (52).

The class of lipid as well as the degree of unsaturation could also affect the properties of membranes at low temperatures. Polar head groups of lipids may be important, but not much is yet known about the in vivo effects (71). In vitro phosphatidyl choline changed phase at a temperature 30C lower than did phosphatidyl ethanolamine (28).

Phospholipids in general might be more important than other lipids. Chill-hardening of Phaseolus vulgaris for 4 days at 12C caused the unsaturation of phospholipids to increase (146), but changes in unsaturation were not detectable when total lipids were analyzed 146).

Since small differences in fatty acid composition might cause large differences in transition temperature (69), and also since other factors influence phase change (lipid class and sterols), direct measures of lipid phase change may be more indicative of chilling sensitivity than fatty acid composition. One method of measuring membrane lipid phase change directly is by electron spin resonance (ESR) where a spin-labeled probe is infused into a membrane. Using this technique a close correspondence was found between growth and phase state of mitochondrial membranes for several plants (115). Although fatty acid composition was not closely related to freezing sensitivity of wheat seedlings, membrane fluidity was closely associated with sensitivity. Genotypes with the most fluid membranes were most resistent (140). The feeding of linolenic acid to sheep caused the temperature of phase change of mitochondrial membranes to decrease (79).

There is not unanimous support for the hypothesis that chilling injury is the result of lipid phase changes. Bishop et al. (10) found that the degree of unsaturation of chloroplast lipids was so great that no phase changes should occur above 0C. This was true for both chilling sensitive and chilling resistent plants. Bishop et al. (10) also criticized the ESR technique of measuring phase change, the technique which had been used by many investigators to demonstrate phase changes.

The spin labels are bulky and likely to cause disturbances in the membrane. Bishop et al. (10) also found that the fluidity measure varied, depending on the specific spin label which was used.

The hypothesis that lipids change phase from fluid to solid at some chilling temperature is probably an over-simplification. Phase separation may gradually occur as the temperature is lowered, whereby the more fluid lipids congregate together and the less fluid lipids congregate together, resulting in solid and fluid domains in the membrane. Using the fluorescent probe, trans-paranaric acid, evidence was found that phase separation between solid and fluid lipids began at about 10C for for some chilling sensitive species, and was not completed until below 0C (109).

Previous ideas about the interactions between enzymes and lipids at chilling temperatures may also require modification. Lyons and Raison (70) proposed that membrane-bound enzymes have two activation energies, one when the lipid environment is above the transition temperature and the other when the lipids are below the transition temperature. Taking into account the idea that there may be increasing phase separation as temperature is lowered, Wolfe (147) proposed that an enzyme is active only when the surrounding lipids are in the fluid state, and as temperature is lowered, progressively more enzyme molecules are in the solid domains and completely inactive. Wolfe also suggested that some amount of solidified lipids may be required for enzymatic activity. Compressibility of both liquids and solids is negligible, but compressibility of both liquids and solids is perhaps 50

times greater in the areas bordering liquid and solid domains. Thus the temperature range for activity of enzymes may occur only when some lipids are solid.

It is possible that some enzymes may themselves be sensitive to low temperature regardless of whether the enzymes are associated with membranes. Pyruvate orthophosphate dikinase from maize was found to be cold labile, appearing to dissociate into two subunits at 12C (120).

Modification of the tertiary structure might be another type of effect that chilling temperatures have on proteins. The fact that hydrophobic bonding is weaker and that electrostatic interactions are stronger at low temperatures (51) suggests that changes in tertiary structure might be expected. The occurrance of non-linear Arrhenius plots for enzymes not associated with lipids, or with enzymes washed free of lipids, would provide evidence for a direct effect of low temperature on enzymes. Non-linear plots for phosphoenol pyruvate carboxylase were found in tropical species, and treatment with Triton X-100 did not change the slope of the Arrenius plots. This suggested that the enzyme itself and not its lipid environment was cold sensitive (44). The enzyme succinate-cytochrome c reductase from a mutant Drosophila had an abrupt change in Ea at 18C. This is a membrane-bound enzyme which requires an association with lipid for activity. When membrane lipids were extracted from normal flies and mixed with purified enzyme from mutant flies there were still breaks at 18C in Arrhenius plots. When enzyme from normal flies was mixed with lipid from mutant flies no such breaks were observed. Thus the inference is that it is

the enzyme, not the lipid, that is temperature sensitive (131). Similarly, the temperature of the break in an Arrhenius plot of succinate oxidase from sweet potato was not affected when phospholipids with a different temperature of phase transition were rebound to the enzyme (149). A break in an Arrhenius plot of Ca+2-ATPase of the sarcoplasmic reticulum occurred at the same temperature even when 95% of the phospholipids were replaced with a detergent (31) or when enzymes had been reactivated with foreign phospholipids (4).

The above evidence suggests that low temperature can directly affect some enzymes. Evidence was also given to support the idea that lipids in membranes can be directly affected by low temperatures, which in turn affect membrane-bound enzymes. A third possibility is that there are other types of lipid-protein interactions that are affected by low temperature. Yamaki and Uritani (150) proposed that the primary event in chilling injury involves the ability of membrane proteins to bind hydrophobically to phospholipids. Chilling was said to break hydrophobic bonds with a resultant cold denaturation of the enzyme-lipid complex. The temperature at which the bonds are broken depends presumably on the hydrophobic nature of the enzyme as well as on the phase transition temperature of the lipids.

For a number of species there is a temperature below which germination no longer occurs, the temperature being anywhere between 0C and 30C (121). An Arrhenius plot of germination rate of mung and cucumber seeds did not show any breaks at the typical 10-12C--germination simply no longer occurred below some temperature

(122). Protein denaturation was suggested as the explanation of the wide range of temperatures for the low limit for germination and for the fact that these low temperature limits were sharp cut-off points.

Low temperatures could interfere with the bonding forces of nucleic acids and nucleic acid-protein interactions during replication, translation, or transcription. Decreased ribonuclease activity and polysome formation was observed in chilled cotton cotyledons (64), possibly indicating a role for nucleic acids in chilling injury. However, there is little direct evidence to date that nucleic acids are chilling sensitive sites.

In summary there is abundant evidence that membranes are involved in chilling injury. Evidence of membrane involvement comes from studies showing differences in degree of unsaturation of lipids from chilling sensitive and chilling resistent species, and from observations that phase changes occur at chilling temperatures in sensitive plants. The role of lipids may be complex, with phase separations and lipid-protein interactions having a role in chilling injury. There is also some evidence that enzymes can be directly affected by temperature, regardless of an association with membranes. There is little evidence to date that nucleic acids or other molecules are primary sites of chilling injury.

It seems possible that lipids, proteins, nucleic acids, and perhaps other molecules may all be primary sites of chilling injury in any given species. Chilling sensitive species are generally of tropical or subtropical origin so from an evolutionary point of view it is

reasonable to assume that their cellular processes are adapted for optimal functioning at non-chilling temperatures. There would be no particular advantage for these plants to have chilling resistance. In fact cold climate adaptations such as highly unsaturated membranes might make these plants less suited for tropical temperatures. The fact that chilling resistant plants such as wheat, the Brassica species, spinach, lettuce, and peas generally do poorly in the summar heat suggests that there is a negative relationship between chilling tolerance and heat tolerance. The literature does not provide much information on the relationship between cold tolerance and heat tolerance in plants.

## Genetic Bases of Chilling Sensitivity

Much of the research on chilling injury has compared chilling tolerant species with chilling sensitive species. These species comparisons have been helpful in developing ideas about the mechanisms of chilling sensitivity. However, chilling tolerance of crop plants can be improved only if genes for resistance can be incorporated into sensitive plants. Thus from the point of view of genetic improvement of crop plants, it is more important to examine differences within species or between closely related species.

Investigators looking for intra-species differences have most often been concerned with germination at low temperatures. Varietal differences in germination and emergence at low temperatures have been found in maize (12, 36, 37, 45, 88, 89, 110, 139), cotton (21, 24, 67,

74), Phaseolus vulgaris (34, 60), lima beans (138), soybeans (96), tomato (38, 57, 94, 130).

Early growth rate has also been used as a criterion for differentiating chilling tolerant from chilling sensitive crop plants, including rice (1, 97), Phaseolus vulgaris (7), tomato (57), and maize (88).

Several other criteria have been used to differentiate cold sensitive from cold tolerant cultivars or closely related species. Differences have been found in electrolyte leakage from chilled leaves of several Passiflora species adapted to different altitudes (100). Differences have been found in leucine uptake at low temperature for Lycopersicon species adapted to different altitudes. Races adapted to the highest altitudes showed nearly linear Arrhenius plots for leucine uptake, but low altitude types showed increasing slopes, indicating increasingly reduced uptake, below 10C (103). Differences in chlorophyll development at low temperature were noted in races of Lycopersicon hirsutum that differed in chilling sensitivity (101). The capacity of isolated chloroplasts from L. hirsutum to photoreduce ferricyanide following chilling varied between races, with the high altitude types being less affected by chilling (126). Cold adapted alfalfa cultivars had higher photosynthetic rates at 10C than did cultivars adapted to warmer climates. The degree of unsaturation of chloroplast membranes was also higher in the cold adapted types (105).

The number of genes involved in the ability to germinate at low temperature has been dealt with by several investigators. Germination

of maize at low temperatures has been found to be multigenic (45, 48, 71, 106, 110). Similarly, germination of cotton (74), and of Phaseolus vulgaris (34) at low temperature have been found to be multigenic. Inheritance of the ability to germinate at low temperatures in tomatoes was less clear, appearing to be multigenic in some cases (38, 94), but appearing to involve a single gene in another case (16).

Germination at low temperature of maize hybrids was found to be more strongly determined by the maternal parent than the paternal parent (14, 48, 110, 139). The existence of maternal effects in maize suggests an involvement of the maternally derived seed coat and/or the endosperm since 2/3 of the endosperm is maternally derived. The maternally derived cytoplasm in the embryo might also play a role in influencing germination at low temperature. Seedling growth in maize appeared to be controlled primarily by additive and dominant gene effects (77). Little or no information is available on the inheritance of any other traits related to chilling sensitivity.

There has been little research on the genetic relationships between two or more chilling sensitivity traits. The presence or absence of linkage between various chilling sensitivity traits is an important consideration for two reasons. First, plant breeders looking for chilling tolerance should know whether chilling injury involves one primary trait or whether there are several genetically independent chilling sensitivity traits. Secondly, the discovery of linkage or of independence of chilling sensitivity traits should give physiologists clues to the primary events involved in chilling injury. The existence

of several genetically independent chilling sensitivity traits would imply the presence of several chilling sensitive sites.

Some investigators have obtained evidence to suggest that chilling sensitivity involves more than one trait. No apparent correlation was found between the temperature at which 50% germination occurred and the temperature at which 50% pollination occurred in several races of Lycopersicon hirsutum adapted to various altitudes (99).

Breaks at about 12C were obtained in Arrhenius plots of respiration, time to 50% germination, and hypocotyl extension rate for several species of Sorghum. However, chlorophyll development was drastically reduced below about 16C. The two different critical temperatures, 16C and 16C, for these traits suggested that there may be at least two different chilling sensitive sites (80).

When cultivars of Phaseolus vulgaris were ranked for seed germination at 8.5C and for fruit set with a night temperature of 4.5C, a completely different ranking order was found for the two traits, suggesting that they were genetically independent (58), although perhaps the same ranking would have been obtained if fruit set and seed germination had been taken at the same temperature.

A break at 12C was observed in an Arrhenius plot of respiration of cucumber leaves. However, severe electrolyte leakage from chilled leaves became apparent only below 8C. The suggestion was made that two sensitive membrane systems were involved, the mitochondrial membrane being affected at 12C, and the plasmalemma being affected at 8C. Another possibility is that a greater extent of solidified lipids was

required for electrolyte leakage than for adverse effects on respiratory enzymes in the mitochondrial membrane (85).

In a mutant barley, chlorophyll synthesis was severely reduced at chilling temperatures but growth rate of the mutant at the low temperatures was not different from a non-mutant barley (125), suggesting that chlorophyll synthesis and growth at low temperatures were independent traits.

In summary, intra-species differences have been found for several traits related to chilling tolerance including germination, early seedling growth, electrolyte leakage, uptake of leucine, chlorophyll development, photosynthetic processes, and the degree of unsaturation of chloroplast membranes. Inheritance studies have been conducted primarily on germination at low temperature and have usually shown multigenic inheritance and maternal effects. Several studies provide evidence that chilling tolerance may involve two or more genetically independent traits.

Typically, researchers have attempted to differentiate chilling tolerant from chilling sensitive lines by use of a single trait. The existence of many genetically independent chilling sensitivity traits would imply that selection based on a single trait may not produce plants that are necessarily generally chilling tolerant.

If individual enzymes are primary sites of chilling injury, then one would expect that chilling sensitive traits would be inherited independently since each enzyme is coded individually by a single gene (or perhaps a few genes in some cases). If lipids are the primary sites

of chilling injury, then the existence of a particular degree of membrane fluidity could affect many enzymes and would cause the expression of a number of phenotypic traits related to low temperature. In this case a relatively small number of genes might be responsible for chilling sensitivity or tolerance. However, chilling tolerance could still involve many genes if the fluidities of membranes were inherited separately for the plasmalemma, endoplasmic reticulum, mitochondrial membranes, chloroplast membranes, tonoplast, nuclear membrane, or other organelle membranes. Little, if any research has dealt with the possibility of independent inheritance of fluidities of organelle membranes.

# C H A P T E R  II

## IMBIBITIONAL CHILLING OF MAIZE AND *ZEA DIPLOPERENNIS* SEEDS

### Introduction

Imbibition of cold water by low moisture seeds has led to reduced germination and growth in many crop plants, including maize (14, 23), lima beans (111), cotton (21), *Sorghum* (108), *Phaseolus vulgaris* (112), and soybean (96, 50).

This experiment had three purposes: to determine whether genotypic differences could be found in sensitivity to imbibitional chilling, to compare sensitivity to imbibitional chilling with chilling sensitivity in a previous field experiment which included the same maize inbreds (88), and to compare sensitivity to imbibitional chilling with response to other types of chilling treatments during seedling growth (to be reported in later chapters). Comparisons of genotypic responses to imbibitional chilling, of genotypic differences in performance under cold field conditions, and of genotypic differences to chilling treatments during seedling growth are important in order to determine whether chilling sensitivity is one general trait or whether chilling sensitivity is a multifaceted phenomenon involving several genetically independent traits.

## Materials and Methods

Plant material. Four inbred lines of maize were obtained from J. J. Mock, Iowa State University. These four lines were four of 34 maize inbreds tested by Mock and McNeill (88) for cold tolerance. B73 and (V3xB14)-2-1 were the two most tolerant and A502 and Mp305 were among the four most sensitive when planted at two locations in Iowa in early April. Seed of B73 and (V3xB14)-2-1 was increased in South Deerfield, Massachusetts. Because of disease problems with A502 and a growing season that was too short for Mp305, additional seedstocks for these 2 inbreds had to be obtained from the Illinois Seed Foundation and Gene Scott, USDA, Mississippi, respectively. Several hybrids were generated at South Deerfield from the 4 inbreds: B73xA502, B73xMp305, Mp305xB73, and B73x(V3xB14)-2-1. In addition a few seeds of Z. diploperennis were obtained from John Doebley, University of Wisconsin, and were increased under greenhouse conditions.

Imbibitional Chilling. Seeds were dried to 5-6% moisture by incubation at 22C for 10-14 days in a desiccator which contained a beaker of 50% (V/V) sulfuric acid (96). After drying, seeds were immersed in water at 2C for 48 hours, or at 22C for 24 hours. Seeds were then planted in moist vermiculite at 22C. Percent emergence was recorded 10 days later. Due to insufficient seed there were no control conditions for chilling of seeds of 15% moisture, nor for the effects of 5% moisture on germination of non-chilled seeds, except for the single inbred, A502. A preliminary experimented indicted that germination of A502 at 22C of seeds initially dried to 5% moisture was not reduced in comparison to

seeds of 15% initial moisture. Cal and Obendorf (14) also found that reduction of moisture to 5% did not reduce germination of maize seeds at a non-chilling temperature; reduced germination occurred only for the combination of 5% initial moisture and imbibition at 5C.

## Results and Discussion

Prior to drying, seeds contained 14-16% moisture. Percent germination of non-dried, non-chilled seeds is recorded in Table 1. The number of seeds germinating per 50 seeds was measured for the imbibitional chilling treatment. In order to obtain an estimate of variance, there were four replicates for each genotype. Mean percent germination of the chilled seeds is presented in Table 1. An analysis of variance was performed on the data after an arcsine transformation, a transformation which is used for counts and percentages (66). The transformation was necessary because data of this type follows a binomial distribution rather than a normal distribution. There were large differences between genotype in percent germination, ranging from 1% for Mp305 to about 93% for the hybrid B73xMp305. Although there was a large effect of heterosis, both of the hybrids which had Mp305 as a parent (male in one case, female in the other) germinated more poorly than did the other two hybrids. These results imply that imbibitional chilling sensitivity is a genetic trait and that poor germination of one parent can be correlated with relatively poor germination in the F1 hybrid.

Table 1. Percent Germination of Non-chilled Seeds of 14-16% Moisture and Percent Germination of Seeds Dried to 5-6% Moisture with Imbibitional Chilling at 2-3C for 48 Hours.

| Genotype | Treatments | |
|---|---|---|
| | Non-chilled | Chilled |
| Mp305 | 86 | 1.0 a [1] |
| A502 | 83 | 30.5 b |
| B73 | 87 | 32.5 b |
| (V3xB14)-2-1 | 90 | 58.0 c |
| B73xMp305 | 89 | 61.0 c |
| Mp305xB73 | 94 | 11.0 a |
| *Zea diploperennis* | 31 | 64.0 c |
| (V3xB14)-2-1xA502 | 95 | 85.0 d |
| B73x(V3xB14)-2-1 | 92 | 92.5 d |

[1] Percentages followed by the same letter are not different ($P<.05$) in a Duncan's multiple range test.

Percent germination of Mp305 in the imbibitional chilling test (Table 1) and also under cold field conditions (88) was very poor, suggesting that response to imbibitional chilling may be one of the reasons why some genotypes germinate poorly in cold soils. However, when the performance of B73 in the imbibitional chilling test and in the field experiment are compared, it appears that germination in the field may involve more than simply imbibitional chilling effects. In the field experiment, B73 had an 88% germination rate (88) and was superior to (V3xB14)-2-1, but in the imbibitional chilling test, germination of B73 was only about 33%, much less than for (V3xB14)-2-1. Response to imbibitional chilling and germination at low temperatures may be independent events, as suggested by Simon (121), and may have separate genetic bases. Thus, germination under cold field conditions may involve chilling injury during imbibition or during subsequent germination or during both, depending on when cold soils occur.

It was thought that the sensitivity of Mp305 to imbibitional chilling might have occurred because seeds were grown in a different climate (Mississippi) and perhaps stored under different conditions. As a check for this possibility a small number of seeds of Mp305 were produced in the greenhouse during the winter of 1980. Seeds produced in this manner were also quite sensitive to imbibitional chilling, 0 of 30 seeds surviving imbibition at 2C for 48 hours. Twenty-four of 30 seeds germinated at 22C.

Although the germination rate of the reciprocal hybrids B73xMp305 and Mp305xB73 were similar at 22C and 15% moisture, the germination rates following cold imbibition of dried seeds showed a large difference

between the two hybrids. Germination of Mp305xB73 was severely reduced by the low temperature imbibition, just as was its female parent. The existence of a maternal effect is in agreement with previous studies with maize (14, 48, 110, 139).

The reduced germination following imbibitional chilling of Zea diploperennis, in contrast to the absence of an effect of chilling on two of the maize hybrids, implies that this species would not be a good source of genes for resistance to imbibitional chilling.

The results of an unplanned experiment provide interesting information about the role of low temperature during maturation of seed. The season at South Deerfield, Massachusetts, was too short for the inbred Mp305, so that freezing temperatures occurred before the seed was completely dry in the field. Inbred seed from these plants germinated poorly at any temperature (that is why seeds of Mp305 had to be obtained from Mississippi). However, seed of the hybrid Mp305xB73, which of course was produced on the same parent, Mp305, and at the same time, did not show reduced germination at 22C even though many seeds appeared abnormally wrinkled. This heterotic effect suggests that in terms of germination at 22C, the embryo of the inbred was the chilling (or freezing) sensitive site during seed maturation because the seed coat is entirely maternally derived and the genetic material of the endosperm is 2/3 maternally derived. However, the occurrance of a lower percent germination of Mp305xB73 than for B73xMp305 following imbibitional chilling implies that the maternally derived parts of the seed, rather than the embryo, are the sensitive sites during imbibition.

The either/or nature of germination following imbibitional chilling is an intriguing phenomenon. Reduced percent survival might be caused by random occurrence of infection by pathogens in the damaged seed. However, the repeatability of the percent emergence in the present experiment argues against a primary role for pathogens because the population of pathogens would be expected to increase from one replication to the next. Simon (121) proposed that reduced survival following imbibitional chilling is due to solute leakage from seeds. However, the question would still remain as to why some seeds germinate while other seeds of the same genotype do not germinate.

# C H A P T E R  III

## GROWTH AND CHLOROPHYLL CONTENT OF PLANTS GROWN AT CONTINUOUS LOW TEMPERATURES

### Introduction

According to Mock and Erbach (87) maize genotypes that grew vigorously and germinated well in cold soils produced the highest grain yields. Several investigators have found genotypic differences in seedling growth at low temperature (86, 88, 89). Thus it appears that rapid growth at low temperatures is a desirable trait and that there is enough variation in this trait to suggest the possibility of improvement of cold sensitive cultivars.

However, rapid growth at low temperature is only one cold tolerance trait. In young seedlings, chilling temperatures could retard or even permanently damage the photosynthetic apparatus without apparent effects on growth while the seedling is still dependent primarily on seed reserves. Maize seedlings grown at 21.8C were primarily dependent on the endosperm for energy during the first 10 days of germination (25). Under cold field conditions, the length of time that the seedling is dependent on the endosperm could be greatly extended because of slower growth. Adverse effects of chilling on the photosynthetic apparatus might not affect growth for perhaps 2-3 weeks or longer.

One type of effect of low temperature on the photosynthetic apparatus that has been observed is to reduce the leaf chlorophyll

content (2, 81, 124, 129). Chlorophyll content at low temperature was found to be closely correlated to photosynthetic rate in maize (136). The present study was concerned with growth rate and chlorophyll content of several maize genotypes grown at low temperatures, and the genetic relationship between growth rate and chlorophyll content was investigated.

## Materials and Methods

Plant Material. Four inbred lines of maize were used. The sources of seed were as noted in Chapter II. These four lines were included with 34 maize inbreds that had been tested for cold tolerance under field conditions (88). B73 and (V3xB14)-2-1 were the two most tolerant and A502 and Mp305 were among the four most sensitive when seed was planted in early spring.

Several hybrids were produced at South Deerfield and included in the experiments: B73xA502, B73xMp305, Mp305xB73, B73x(V3xB14)-2-1, and (V3xB14)-2-1xMp305. The wild plant referred to as perennial maize, Zea diploperennis, was included in some of the experiments. The sources of seed were as specified in Chapter II. A few seeds from the cross B73xZea diploperennis were used in one of the chlorophyll experiments. Seed for this interspecies hybrid was made from a cross performed under greenhouse conditions.

Growth Conditions. Plants were grown either in a growth chamber or a temperature-controlled room, both equipped with cool-white fluorescent lights and both at an intensity of 750 ue/m2/sec and with a photoperiod

of 14 hours of light and 10 hours of darkness. Humidity was not controlled and ranged from 75% to 85%. The growth room was used for the control treatments requiring 22C, and the growth chamber was used for all lower temperatures. Plants were grown in vermiculite in plastic 4"x6" containers. Plants were watered every 1-2 days with Hoagland's solution modified to provide triple the concentration of iron. At each watering a sufficient quantity was used to cause a small amount of solution to leach through the containers. Prior to planting in vermiculite seeds were soaked one day in tap water, a treatment which produced more uniform germination than when dry seeds were planted.

Growth was measured as increase of either dry weight or length of shoots. Length was the average of the two longest leaves from tips down to the node just above the seed where the highest adventitious root occurred. Shoots were dried to constant weight at 70C for approximately 48 hours in a forced air dryer.

Chlorophyll Determinations. Chlorophyll was extracted in 80% aqueous acetone from whole shoots. Chlorophyll of the extracts was measured by absorption spectroscopy at a wavelength of 652nm and the method of Arnon (5) was used to calculate chlorophyll which is expressed as mg chlorophyll per gram fresh weight.

## Results and Discussion

Comparison of the genotypes for growth rate at 16C was an important objective of the experiment. Additionally, a measure of the interaction

between genotype and temperature was also an important consideration. Thus the statistical analysis incorporated two temperature treatments: plants grown at a constant 16C for 30 days, and plants grown at a constant 22C for 10 days. The longer growth period for the plants held at 16 was used for two reasons. First, it was necessary because the slowest growing genotypes had not completely emerged even at 3 weeks. Second, it was desired to compare chlorophyll of cold grown plants and control plants of similar size. Preliminary experiments had shown that shoot dry weight production was similar under these two treatments.

Results of the analysis of variance are presented in Table 2. The treatment effect was not significant ($p>.05$) confirming the observation that there was about the same amount of growth after 10 days at 22C than there was after 30 days at 16C. There were highly significant differences both for genotype and for treatment x genotype interaction. A Duncan's multiple range test was performed for each treatment separately and is presented in Table 3. The data demonstrate the effect of heterosis for growth at 16C since all three hybrids were larger than their parents. The performance of the inbreds is in general agreement with a previous field experiment where these inbreds were planted early at two locations and dry weight was taken after 30 days (88). Despite the dissimilarily between growth at a constant 16C and the fluctuating temperature conditions in the field, a similar relationship among inbreds was observed: dry weight production of B73 and (V3xB14)-2-1 was greater than dry weight production of Mp305 and A502. These results, in conjunction with the results showing similar growth of Mp305 and B73 at

Table 2. Analysis of Variance for Dry Weight Production of 7 Genotypes and 2 Temperature Treatments: 30 Days at 16C and 10 Days at 22C.

| Source | df | Mean Square | F |
|---|---|---|---|
| Treatments | 1 | .000626 | 3.56 ns |
| Genotype | 6 | .056605 | 322 [1] |
| Treatment x Genotype | 6 | .011588 | 65.9 [2] |
| Error | 322 | .000176 | |

[1] Significant at $p<.005$.

[2] Significant at $p<.01$.

Table 3. Dry Weight Production (Grams) for 7 Maize Genotypes Grown at 22C for 10 Days and at 16C for 30 Days.

| Genotype | 30 Days at 16C | 10 Days at 22C |
|---|---|---|
| (V3xB14)-2-1xMp305 | .142(.021) a [1] | .121(.016) a |
| B73x(V3xB14)-2-1 | .124(.022) b | .095(.010) b |
| B73xMp305 [2] | .110(.021) c | .072(.001) c |
| B73 | .069(.015) d | .073(.008) c |
| (V3xB14)-2-1 | .045(.010) e | .087(.009) b |
| Mp305 | .030(.005) f | .068(.012) c |
| A502 | .022(.004) f | .041(.008) d |

[1] Values in parentheses are standard deviations. Within each treatment, values not sharing a letter are significantly different ($p<.05$) in a Duncan's multiple range test.

[2] The reciprocal hybrids Mp305xB73 and B73xMp305 were not different from each other in either treatment.

22C, imply that there are significant genotypic differences in sensitivity to low temperature and that the relative sensitivity of each genotype is similar under either constant or varying low temperature conditions.

The sources of the significant interaction between genotype and treatment can be seen by comparing growth at the two temperatures. Among the inbreds, B73 grew faster than (V3xB14)-2-1 at 16C, while (V3xB14)-2-1 grew faster than B73 at 22C. Mp305 grew as well as B73 at 22C, but grew much slower at 16C. A temperature-dependent heterosis appears also to have contributed to the genotype x temperature interaction. At 22C the hybrid B73xMp305 grew at the same rate as B73, but at the lower temperature the hybrid grew faster than any of the inbreds.

A lack of a significant difference between the reciprocal hybrids B73xMp305 and Mp305xB73 argues against any maternal effect on growth for this particular cross.

Although B73 was the fastest growing inbred when dry weights were measured after 30 days at 16C, (V3xB14)-2-1 grew faster than B73 for the first two weeks at 16C (Table 4). In this case growth was measured as length rather than as dry weight. The correlation between these two measures of growth, dry weight and length, was .96 after 30 days at 16C.

Chlorophyll content of plants grown at 16C is shown in Table 5. All of the inbreds were quite low in chlorophyll when compared to the hybrids or whe n compared to chlorophyll content of plants grown at 22C. There were significant differences between the inbreds. The inbred

Table 4. Shoot Length of 4 Maize Inbreds after 14 Days at 16C.

| Genotype | Shoot Length |
|---|---|
| (V3xB14)-2-1 | 5.16(.94) a [1] |
| B73 | 2.16(1.11) b |
| Mp305 | .42(.56) c |
| A502 | .37(.59) c |

[1] Values in parentheses are standard deviations. Values not sharing a leter are significantly different ($p<.05$) in a Duncan's multiple range test.

Table 5. Chlorophyll Content of Shoots after 30 Days at 16C and 10 Days at 22C.

| Genotype | 30 Days at 16C | 10 Days at 22C |
|---|---|---|
| Zea diploperennis | 1.24(.26) a [1] | 1.11(.17) |
| B73xMp305 | 1.24(.05) a | 1.14(.08) |
| Mp305xB73 | 1.19(.09) a | --- |
| B73x(V3xB14)-2-1 | .93(.07) b | .99(.08) |
| (V3xB14)-2-1xA502 | .67(.05) c | .88(.06) |
| (V3xB14)-2-1xMp305 | .66(.08) cd | --- |
| B73 | .59(.07) d | 1.04(.12) |
| A502 | .46(.07) e | 1.11(.12) |
| Mp305 | .25(.09) f | 1.06(.09) |
| (V3xB14)-2-1 | .09(.01) g | .95(.07) |

[1] Values in parentheses are standard deviations. Values not sharing a letter are significantly different (p.05) in a Duncan's multiple range test.

(V3xB14)-2-1, which was the fastest growing inbred at 16C for the first 14 days (Table 4) and was also a cold tolerant inbred in a field test (88) had the lowest amount of chlorophyll. Thus it seems likely that the chlorotic (V3xB14)-2-1 maintained its ability to grow rapidly at 16C as long as seed reserve was available, but its growth after 30 days at 16C (Table 3) was limited by a lack of photosynthesis.

In general the hybrids were much less chlorotic than the inbreds, indicating a strong effect of heterosis for this trait. (V3xB14)-2-1 was a parent for three hybrids: B73x(V3xB14)-2-1, (V3xB14)-2-1xA502, and (V3xB14)-2-1xMp305. These three hybrids which all have the most chlorotic parent, (V3xB14)-2-1, in common were lower in chlorophyll than the other two hybrids, suggesting that the genes which limit chlorophyll content in the inbreds are also expressed in the hybrids. There is additional evidence that genes in the inbreds were being expressed in the hybrids. A cross between the two most chlorotic inbreds, (V3xB14)-2-1xMp305, was more chlorotic than was the cross between (V3xB14)-2-1 and the least chlorotic inbred, B73. Thus, although considerable heterosis was involved, chlorophyll content in the parents was associated with chlorophyll content in the hybrids. Strong dominance effects were not apparent for this trait.

There were no differences between the reciprocal hybrids B73xMp305 and Mp305xB73. Chlorophyll content at 16C therefore does not appear to be maternally inherited for this particular cross.

When chlorophyll content was determined in plants grown at 12C (Table 6) no differences between the inbreds were observed. All of the

Table 6. Chlorophyll content of Shoots Grown at 12C for 40 Days.

| Genotype | Chlorophyll (mg/gm fresh weight) |
|---|---|
| B73x*Zea diploperennis* | 1.10(.15) a [1] |
| *Zea diploperennis* | 1.04(.23) a |
| B73xMp305 | .344(.030) b |
| B73x(V3xB14)-2-1 | .245(.020) bc |
| A502xB73 | .214(.034) cd |
| (V3xB14)-2-1xA502 | .192(.012) cd |
| B73 | .136(.024) cde |
| Mp305 | .086(.012) de |
| (V3xB12)-2-1 | .071(.010) e |
| A502 | .065(.007) e |

[1] Values in parentheses are standard deviations. Values not sharing a letter are significantly different ($p<.05$) in a Duncan's multiple range test.

maize hybrids were quite low in chlorophyll at this temperature when compared to the levels at 16C or 22C (Table 5). However, the species Zea diploperennis was quite green, its chlorophyll content at 12C being similar to the level of that in plants grown at 16C or 22C. The large standard deviation for Zea diploperennis is not unexpected because this species was grown for only a few generations in the greenhouse since it was collected from the wild, and thus segregation for this trait may have been occurring. Each of the maize inbreds and hybrids was of course genetically uniform.

The cross B73xZea diploperennis contained as much chlorophyll at 12C as did Zea diploperennis. Thus it may be possible to incorporate resistence to low temperature chlorosis from Zea diploperennis into maize genotypes.

The possibility was considered that rapid growth rate and low chlorophyll content at 16C were positively correlated because (V3xB14)-2-1 was both the most rapid growing inbred and the most chlorotic. The relationship between growth and chlorophyll content was investigated by using a population of F2 plants of the cross (V3xB14)-2-1xA502 that was grown at 16C. After three weeks of growth the correlation between shoot length and chlorophyll content was low (.136) and non-significant. Therefore the chlorotic plants did not grow faster. It appears that resistance to chlorosis at 16C could be incorporated into sensitive inbreds without detrimental effects on early growth.

It is possible that chlorosis is not an important consideration in the development of chilling resistant maize cultivars. One the one hand high light intensity in conjunction with low temperature was shown to produce chlorosis (9, 81, 136), and these conditions would often occur in early spring under field conditions. On the other hand field temperatures fluctuate. Brief warm periods might be sufficient to prevent chlorosis or to allow rapid regreening of chlorotic tissue. To answer the question of the rapidity of regreening of chlorotic tissue, the inbred (V3xB14)-2-1 was grown for two weeks at 18C/14C (light/dark) and with a photoperiod of 14 hours of light and 10 hours of darkness. This treatment still produced quite chlorotic tissue, as seen at Day 0 in Figure 1, although not quite as chlorotic as had been produced by a constant 16C (Table 5). Chlorophyll content of the second and third leaves was measured at 0, 1, 3, and 7 days after transfer to a constant 22C. There was a gradual greening of tissue, but even after one week chlorophyll content of the second and third leaves did not reach the concentration found in whole shoots grown at a constant 22C. At one week after transfer to 22C the leaves did not appear normal but had streaks and diffuse patches of chlorotic tissue. Thus, regreening of chlorotic tissue does occur, but the process was not rapid.

The question of whether chlorosis occurs at all under field conditions could be answered simply by planting early and comparing chlorophyll content of genotypes which were shown to differ in chlorophyll content at a constant 16C, for example, B73 vs (V3xB14)-2-1.

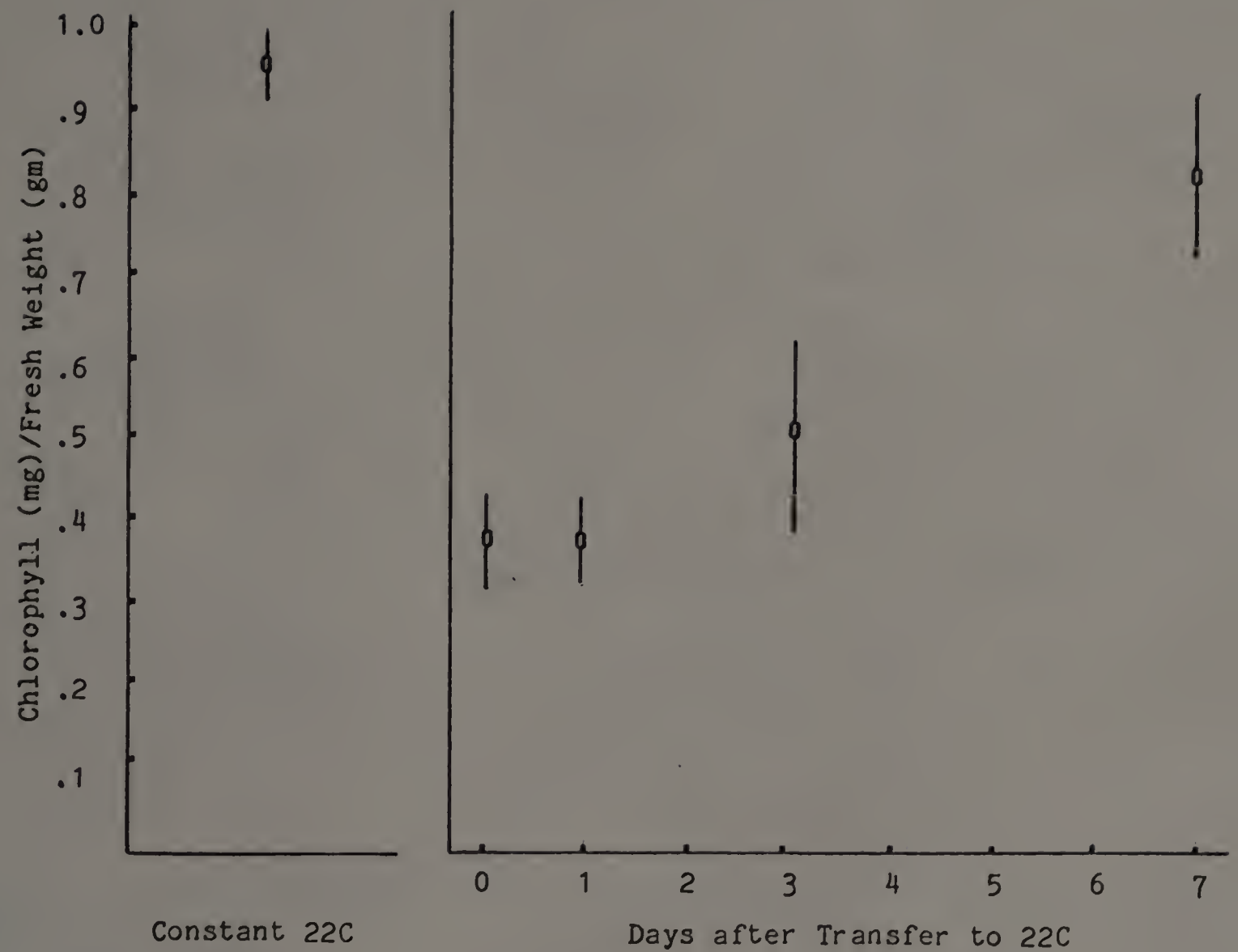


Figure 1. Chlorophyll content of second and third leaves of (V3xB14)-2-1 grown at 18C in the light and 14C in the dark with a photoperiod of 14 hours of light and 10 hours of darkness. Plants were assayed for chlorophyll content at 0, 1, 3, or 7 days after transfer to a constant 22C.

Chlorophyll content at a constant 22C was taken from Table 5 and represents chlorphyll content of whole shoots at 10 days. Vertical lines through the points represent standard deviations. The means of 5 plants were used in each determination of chlorophyll.

Assuming chlorosis does occur under cold field conditions, a definitive test of the role of chlorophyll in chilling sensitivity would be to develop by backcrossing with Zea diploperennis a (V3xB14)-2-1 which is green when grown at 16C or lower. Then growth of the green (V3xB14)-2-1 and the old, chlorotic, (V3xB14)-2-1 would be compared after early plantings at several locations.

The results of the present experiments show that growth of seedlings and chlorophyll content at 16C were genetically independent traits. Thus, in developing cold tolerant maize it might be worthwhile to select for rapid growth as well as for high chlorophyll content at low temperatures. However, the importance of chlorophyll content of young seedlings under field conditions remains to be determined. Zea diploperennis might be a good source of genes for resistance to low temperature chlorosis, although this species did not appear to have any advantage over maize in ability to grow at low temperature. The slow growth of Zea diploperennis may be partly due to the much smaller seed size and to the fact that much of the small seed appears to be a non-nutritive protective coat. A much smaller seed reserve would imply that seed reserve is soon exhausted and the plant must then depend entirely on photosynthesis as a source of energy.

# C H A P T E R  IV

# EFFECTS OF CHILLING ON GROWTH OF PLANTS ESTABLISHED AT A NON-CHILLING TEMPERATURE

## Introduction

It has been well documented that maize is sensitive to chilling temperatures during imbibition (14, 23) and that germination of maize is reduced at low temperatures (36, 37, 45, 12, 83). However, there is surprisingly little data on the effects of chilling on maize seedlings that have been established under non-chilling temperatures. Electron spin resonance probes have revealed a phase change at about 12C in lipids from maize root mitochondria, and Arrhenius plots of succinate oxidase from maize root mitochondria have revealed a break at the same temperature (116). This suggests that established maize seedlings may be sensitive to temperatures below 12C. A respiratory increase was noted in maize leaves following a very severe chilling treatment, 0.3C for 48 hours (26). The present experiments explore the effects of two chilling treatments, 2-3C and 10-11C, on the growth of maize seedlings that had been established at 22C. The existence of genotypic differences in sensitivity to these chilling temperatures was explored.

## Materials and Methods

Plant Material. The maize genotypes included the four inbred lines previously tested, A502, B73, Mp305, and (V3xB14)-2-1. The hybrid (V3xB14)-2-1xA502 was also included and represents a cross between the slowest and fastest growing inbreds (Table 2). The species Zea diploperennis was also included and sources of seed were as specified in Chapter II.

Growth Conditions. Plants were grown under light and nutrient conditions as specified in Chapter III.

Chilling Treatments. The experimental design included the six genotypes and two treatments: 1) a control group whose dry weight was taken after 14 days of growth at 22C, and 2) a chilled group. There were two types of chilling treatment, one consisting of plants which were grown one week at 22C, then one week at 10-11C, then an additional week at 22C. The other chilling treatment consisted of plants grown one week at 22C, then chilled 48 hours at 2-3C in the dark, then grown an additional week at 22C.

The choice of 14 days as the growth period for the control group was arbitrary in that the control group was used only for comparing growth of genotypes at 22C and as a means of deriving the ratio chilled/control for comparing genotypes. A period of 21 days could have been used for the control group as it was for the chilled plants; however, control plants of this age were too unwieldly for the growth chamber.

The experimental design incorporated 5 blocks per treatment and 4 plants of each genotype per block. Containers of plants represented

blocks in the statistical analysis, with each container including all 6 genotypes. In the statistical analysis blocks were nested within treatments.

## Results and Discussion

An analysis of variance for dry weight production is presented in Table 7 for the 10-11C chilling treatment. There was a significant genotype x treatment interaction. The means, standard deviations and results of a Duncan's multiple range test are presented in Table 8. Within both treatments all genotypes were different from each other except for B73 and Mp305, which were not significantly different from each other within either the control group or the chilled group. However, the relative ranks of B73 and Mp305 were opposite in the two groups, B73 being higher in the chilled group and Mp305 being higher in the control group.

The relative effects of the chilling treatment can be seen in the last column of Table 8, where the ratios of the mean dry weight productions are listed for the chilled treatment divided by the control. Much of the interaction between genotype and treatment came from two sources, the reduced growth of Mp305 in the chilled treatment and the increased growth of the hybrid in the chilled treatment.

Dry weight of chilled/control for *Zea diploperennis* does not indicate that this species was more resistant to the chilling treatment than were some of the maize genotypes.

Table 7. Analysis of Variance for Dry Weights for 6 Genotypes Grown at 22C for 14 Days (Control), and for Plants which were Grown for 7 Days at 22C Followed by 7 Days at 10-11C Followed by a Final Week at 22C.

| Source | df | Mean Square | F |
|---|---|---|---|
| Treatments | 1 | .0194 | 7.52 [1] |
| Blocks | 8 | .00258 | no test |
| Genotypes | 5 | .366 | 458 [2] |
| Genotypes x Treatments | 5 | .00501 | 6.26 [1] |
| Genotypes x Blocks:Treatments | 40 | .000801 | 1.45 ns |
| Within Cells | 163 | .00055 | |

[1] Significant at $p<.05$.

[2] Significant at $p<.01$.

Table 8. Mean Dry Weights of Plants grown for 14 Days at 22C (Control) and of Chilled Plants (Grown 7 Days at 22C, Chilled 7 Days at 10-11C, and Returned to 22C for 7 Days).

| Genotype | Control | Chilled | Chilled/Control |
|---|---|---|---|
| A502x(V3xB14)-2-1 | .252(.034) a[1] | .312(.037) v | 1.24 |
| (V3xB14)-2-1 | .235(.036) b | .250(.037) w | 1.06 |
| B73 | .122(.019) c | .130(.014) x | 1.07 |
| Mp305 | .129(.014) c | .114(.037) x | .88 |
| A502 | .068(.010) d | .076(.010) y | 1.12 |
| *Zea diploperennis* | .026(.005) e | .030(.010) z | 1.15 |

[1] Values in parentheses indicate standard deviations. Values within control or within chilled treatments which do not have a letter in common are significantly different ($p<.05$) in a Duncan's multiple range test.

When the 2 treatments were compared the only significant effect was for the hybrid (V3xB14)-2-1xA502, although the mean dry weights for all of the other genotypes except Mp305 were slightly higher in the chilled treatment than in the control. The failure of most genotypes to produce significantly more dry weight in the three weeks of the chilled treatment than in the two weeks of the control treatment may be due to either little or no growth during the week at 10-11C, or to the occurrance of some growth at 10-11C but also with some chilling injury which reduced growth during the final week at 22C.

The data indicate that the chilling treatment of one week at 10-11C did not produce large differences among the inbreds when the effect of chilling was expressed as a ratio of dry weight of chilled plants/dry weight of control plants. Thus, an experiment incorporating a more severe chilling treatment was devised. The 6 genotypes were grown for one week at 22C, then chilled for 48 hours at 2-3C, and returned to 22C for an additional week. Relative humidity was 90-95% during chilling. The ratio of growth of chilled plants to non-chilled plants was determined for each genotype. The mean of 4 chilled plants was divided by the mean of 4 non-chilled plants for each genotype. To obtain an estimate of variance for the ratios, the experiment was repeated 5 times, so that in the analysis of variance there were 6 genotypes with 5 replicates each. Means for dry weight for the chilled plants and for the ratios are presented in Table 9.

The dry weights of the chilled plants fell into the same rank as was observed in the previous experiment where plants were grown for 14 days

Table 9. Mean Dry Weights of Plants Grown 14 Days at 22C (Control) and Chilled Plants (Grown 7 Days at 22C, Chilled at 2-3C for 48 Hours, and Returned to 22C for 7 Days).

| Genotype | Dry Weight | Chilled/Control |
|---|---|---|
| A502 | .063(.014) | .916(.081) a [1] |
| B73 | .091(.018) | .744(.067) b |
| Mp305 | .087(.026) | .725(.079) b |
| *Zea diploperennis* | .017(.002) | .660(.067) bc |
| A502x(V3xB14)-2-1 | .143(.032) | .577(.065) cd |
| (V3xB14)-2-1 | .128(.031) | .549(.046) d |

[1] Values not having a letter in common are significantly different ($p<.05$) in a Duncan's multiple range test. Values in parentheses are standard deviations.

at 22C (Table 8). When the effect of chilling was computed as a ratio, the ranking for the inbreds was reversed. At one extreme, the inbred A502 grew about 92% as well after being chilled as did non-chilled plants, while at the other extreme (V3xB14)-2-1 grew only about 55% as well after chilling. The hybrid (V3xB14)-2-1xA502 was not significantly different from the parent (V3xB14)-2-1. Zea diploperennis was no more resistent to the chilling treatment than were four of the five maize genotypes.

Thus the chilling treatment of 2-3C for 48 hours was very effective in producing genotypic differences. In addition to reducing growth rate the chilling treatment also produced necrotic tissue on the leaves. More about these symptoms will be said in Chapter IV. Factors influencing the response to chilling at 2-3C will also be discussed in Chapter IV.

# C H A P T E R  V

# EFFECTS OF GENOTYPE, PLANT SIZE, ROOT TEMPERATURE AND RELATIVE HUMIDITY ON THE SEVERITY OF INJURY FOLLOWING CHILLING AT 2C

## Introduction

In Chapter IV data were presented that indicated large genotypic differences in relative growth rates following chilling at 2-3C for 48 hours. It was also noted that leaves developed necrotic tissue following chilling. In the present experiments the role of several variables in influencing the severity of injury following chilling at 2C was investigated.

## Materials and Methods

Chilling treatments involved 2-3C and were applied after 7 days of growth as in Chapter IV. One measure that was used in several of the experiments to estimate the relative effects of chilling was a ratio of dry weight to fresh weight taken one week after cessation of the chilling treatment. This period of time following chilling was sufficient to allow necrotic tissue to dry out. Thus, a large dry weight/fresh weight ratio indicates extensive necrotic tissue and/or a small amount of growth following chilling. Light intensity and photoperiod during the week prior to and the week following chilling

were the same as specified in Chapter III. Chilling periods were in the dark and the duration was either 12 or 48 hours.

## Results and Discussion

The first experiment measured the effects of chilling at 2-3C for 48 hours on the 9 genotypes. The dry weight/fresh weight ratios presented in Table 10 indicate significant differences between genotypes. When the mean dry weights of the inbreds are compared with the effects of chilling on growth (Table 9) and on the dry weight/fresh weight ratios (Table 10) it appears that there is a relationship between plant size and the sensitivity to chilling. The slowest growing inbred, A502, was much less affected than the fastest growing inbred (V3xB14)-2-1. Similarly, the results in Chapter IV for dry weight production of chilled plants divided by the dry weights of the control plants (Table 9) reveal that in general the larger genotypes were relatively more affected by chilling than were the smaller genotypes. There are at least two possible explanations for this relationship: that rapid growth rate and susceptibility to chilling are directly related or that larger plants are simply more vulnerable to this treatment. The latter explanation would imply that slow growing inbreds would be just as sensitive when the plants reached a larger size.

To test the hypothesis that plant size is one factor which determines the amount of necrotic tissue that develops as a consequence of chilling, A502 (the median genotype in Table 10) was chilled at 7, 9,

Table 10. Dry Weight/Fresh Weight of Plants Grown 7 Days at 22C, Chilled 48 Hours at 2-3C, and Returned to 22C for 7 Days.

| Genotype | Dry Weight/Fresh Weight |
|---|---|
| Mp305x(V3xB14)-2-1 | .086(.005) a [1] |
| Zea diploperennis | .091(.009) ab |
| Mp305 | .092(.004) ab |
| B73x(V3xB14)-2-1 | .097(.003) bc |
| A502 | .098(.005) c |
| (V3xB14)-2-1xA502 | .099(.007) c |
| B73xMp305 | .100(.004) c |
| B73 | .103(.005) c |
| (V3xB14)-2-1 | .122(.010) d |
| Non-chilled plants, all genotypes | .076(.006) [2] |

[1] Values not having a letter in common are significantly different ($p<.05$) in a Duncan's multiple range test. Values in parentheses are standard deviations.

[2] Non-chilled plants were not included in the statistical analysis.

and 11 days. The larger, 11 day old plants were affected much more than the smaller, 7 day old plants (Table 11). The greater susceptibility of the larger and older plants may have been due to morphological changes such as shoot/root ratio, or there may have been physiological changes between 7 and 11 days which made the older plants more sensitive. For example, as leaves of Phaseolus vulgaris aged, a decrease in unsaturation of phospholipids and a corresponding increase in susceptibility to chilling was observed (146). Similar changes might occur in maize leaves.

The standard deviations in Table 11 were larger for the older, more damaged plants. An explanation for this might be that individual plants which have lost the least amount of photosynthetic tissue produce fresh weight faster than the more damaged plants, so that after one week of additional growth the differences become larger than the differences which existed immediately following chilling.

To further test the relationship between plant size and susceptibility, 61 F2 plants of the cross (V3xB14)-2-1xA502 were grown one week at 22C then chilled for 48 hours at 2-3C, and returned to 22C for an additional week. The correlation between plant height prior to chilling and the dry weight/fresh weight ratio one week after chilling was .19. The positive correlation suggests a positive relationship between plant size and susceptibility, but the correlation is not statistically significant. The correlation might have been higher if weak plants had been eliminated before chilling. Preliminary experiments had shown that about 5% of the plants of this F2 cross were weak, small, and developed some necrotic tissue even when grown at 22C.

Table 11. Dry Weight/Fresh Weight of A502 that was Chilled at 7, 9, and 11 Days.

| Age at Chilling (days) | Dry Weight/Fresh Weight |
|---|---|
| 7 | .097(.005) a [1] |
| 9 | .127(.029) ab |
| 11 | .155(.040) b |

[1] Values not having a letter in common are significantly different ($p<.05$) in a Duncan's multiple range test. Values in parentheses are standard deviations.

There was some evidence that plant size was not the only factor in determining dry weight/fresh weight ratios following chilling. The hybrid (V3xB14)-2-1xMp305 was significantly larger ($p<.05$) than the hybrid B73xMp305, but the larger hybrid had the lower dry weight/fresh weight ratio. Additional evidence comes from the fact that the two inbreds Mp305 and B73 were always non-significantly different in size when the plants were grown at 22C (Table 3, Table 8, and Table 10), but Mp305 had a significantly lower dry weight/fresh weight ratio than B73. And, finally, although there was a positive relationship between plant size and susceptibility in the F2 generation of (V3xB14)-2-1xA502, the correlation was small. There were some large F2 plants that had a low dry weight/fresh weight ratio, suggesting that these plants had a genetic based resistance to chilling.

There were no clear inheritance patterns for the susceptibility to chilling at 2-3C. For example, the two hybrids B73xMp305 and (V3xB14)-2-1xMp305 have the male parent Mp305 in common and differ only in the female parent. (V3xB14)-2-1xMp305 was the more resistent hybrid even though (V3xB14)-2-1 was the more sensitive inbred. Thus it seems that a particular cross may involve complementation and that performance of the parent is not a good prediction of hybrid performance for this trait.

Although chilling at 2-3C had a significantly greater effect on B73 than on Mp305 in terms of dry weight/fresh weight ratio (Table 10), there were no significant differences in terms of growth (Table 9). It is possible that the ability to grow following chilling and the amount

of necrotic tissue produced as a result of chilling are separate genetic traits.

The species Zea diploperennis was as sensitive to chilling at 2-3C as were several of the maize genotypes. This was true both in terms of reduced growth (Table 9) and dry weight/fresh weight ratio (Table 10).

The chilling treatment of 48 hours at 2C is not a condition that would ofccur under field conditions. To determine whether a chilling treatment of shorter duration, as might occur in the field, would have a measurable effect either on growth or dry weight/fresh weight ratio, B73 and the hybrid B73xMp305 were chilled for one dark period of 12 hours at 2-3C. Dry weight and dry weight/fresh weight ratios were taken 7 days after return to 22C. This chilling treatment had a significant effect in reducing dry weight of both genotypes (Table 12). Again dry weight production of the larger genotype was more affected by the chilling treatment, as indicated by a significant treatment x genotype interaction ($p<.05$). Dry weight/fresh weight ratio was also significantly affected by the chilling treatment, even though visual observation did not indicate the development of necrosis. The effect on dry weight/fresh weight was small and there was no genotype x treatment interaction. Thus, reduced growth may be a more sensitive measure of chilling injury than the ratio.

In all of the preceding experiments involving a 2-3C chilling treatments, roots as well as shoots were chilled. Under field conditions roots may not be exposed to temperatures as low as shoots because of the ability of the soil to retain heat and because of the

Table 12. Dry Weight and Dry Weight/Fresh Weight of Plants 7 Days after Chilling of 12 Hours at 2-3C Compared with Non-Chilled Controls.

| Genotype | Control | | Chilled | |
|---|---|---|---|---|
| | DW | DW/FW | DW | DW/FW |
| Mp305 | .114(.007) [1] | .073(.002) | .095(.009) | .077(.003) |
| Mp305xB73 | .227(.037) | .069(.001) | .151(.019) | .072(.003) |

[1] Values in parentheses are standard deviations.

insulating properties of the top few inches of soil. Thus the effects of chilling at 2-3C may have been due primarily to effects on the roots. An experiment was designed to determine the effects on chilled shoots when the root zone was maintained at a higher temperature. Two genotypes were chosen, A502, the inbred whose growth was least affected by chilling, and (V3xB14)-2-1, the inbred whose growth was most reduced by chilling (Table 9). Plants were grown in two containers made of 2" styrofoam. On the bottom of one container, loops of 3/8" copper tubing were placed and warm water was pumped through the loops during the 48 hours of chilling at 2C. Aluminum foil was laid on the surface of the vermiculite root medium between rows of plants to promote more uniform root zone temperature. During chilling the root zone was maintained at 13-16 C in the heated container and at 2-3C in a similar container which was unheated. Keeping the roots at 13-16 C had no effect in reducing the dry weight/fresh weight ratios (Table 13). Chilling whole plants had a tendency to reduce dry weight production of one genotype, A502, but the effect was not significant. It is possible that a higher root temperature might have had a protective effect. However, when air temperature is 2-3C in the field, temperatures of lower than 16 C would often occur in the root zone of small seedlings.

There were considerable differences in dry weight/fresh weight ratios between experiments even though the standard deviations for this ratio were typically low in any given experiment. The dry weight/fresh weight ratio of (V3xB14)-2-1 in the above experiment was .087 when the whole plant was chilled, but was .122 in a previous experiment where

Table 13. Dry Weights and Dry Weight/Fresh Weight of Shoots Chilled 48 Hours at 2C with Root Zone Temperatures of 2-3C and 13-16C.

| | Dry Weight | | |
|---|---|---|---|
| Genotype | Control [1] | Chilled Shoots | Chilled Whole Plant |
| A502 | .068(.010) | .067(.018) | .054(.009) [2] |
| (V3xB14)-2-1 | .235(.036) | .132(.031) | .132(.032) |
| | **Dry Weight/Fresh Weight** | | |
| A502 | .076(.006) | .086(.003) | .086(.004) |
| (V3xB14)-2-1 | .076(.006) | .083(.004) | .087(.003) |

[1] Dry Weights of non-chilled controls are taken from Table 9. Dry weight/fresh weight of non-chilled controls were taken from Table 11. The ratio was similar for all genotypes.

[2] Values in parentheses are standard deviations.

the whole plant was also chilled (Table 10). There were some differences in environmental conditions between the two experiments. In the present experiment, plant density was lower and the root zone was deeper. There may have been differences in humidity since humidity was not controlled and fluctuated with outsizde environmental conditions. Slightly altered growth conditions could also have affected plant size which in turn could have affected sensitivity to chilling.

Water stress was found to have a role in determining the extent of chilling injury in Phaseolus vulgaris . A relative humidity of 100% protected plants from the effects of chilling at 5 C (143). To test the effects of relative humidity during chilling of maize the inbred (V3xB14)-2-1 was chilled at 2C for 48 hours, with some plants being chilled inside plastic bags. Bags were removed immediately following chilling and the plants were allowed to grow at 22C for an additional week as in previous experiments. This treatment had no significant effect when compared to plants chilled without plastic bags (Table 14). However, plants left in plastic bags for two hours following chilling had a significantly lower dry weight/fresh weight ratio than did plants chilled without bags. Two hours was a period of time which allowed root zone temperature to equilibrate with air temperature.

The protective effect of the plastic bags suggested that water stress was involved in the development of necrotic tissue. Diffusive resistance measurements of leaves provided evidence that chilling induced a change in stomatal opening or perhaps damage to leaves which led to water loss (Table 15). If at the same time, the chilled roots

Table 14. Dry Weight/Fresh Weight of (V3xB14)-2-1 of Plants Bagged and Not Bagged During and 2 Hours Subsequent to Chilling at 2-3C.

| Treatment | DW/FW |
|---|---|
| Not Bagged | .102(.010) a [1] |
| Bagged During Chilling | .098(.009) a |
| Bagged During Chilling Plus 2 Hours | .081(.003) b |

[1] Values not having a letter in common are significant ($p<.05$) in a Duncan's multiple range test. Values in parentheses are standard deviations.

Table 15. Diffusive Resistance Measurements (sec/cm) of the First Leaves of (V3xB14)-2-1 One Hour Following Chilling at 2-3C for 5 Hours, and of Non-Chilled Plants (Controls).

| Treatment | Resistance |
|---|---|
| Dark, Non-Chilled | 165(35.3) [1] |
| Light, Non-Chilled | 19.5(4.5) |
| Dark, Chilled | 64.5(18.7) |

[1] Values in parentheses are standard deviations.

were not able to absorb adequate water, a water stress would occur in the plant.

Although bagging the plants during chilling plus an additional two hours following chilling had a protective effect, this treatment did not eliminate the development of necrotic tissue. This suggested that water stress was not the only factor in the development of necrotic tissue. Further evidence that water stress was not the only factor was provided by an experiment in which plants were chilled in clear plastic bags and left in the bags for an additional two days following chilling. Control plants were also placed in plastic bags for two days at 22C. Chilling again was 2-3C for 48 hours and occurred after 7 days of growth, and plants were allowed to grow for an additional 7 days after chilling. Results are presented in Table 16. The values for the treatment "Chilled, Not Bagged" were taken from previous experiments, dry weight from Table 9 and dry weight/fresh weight from Table 10. When the bagged, chilled plants were compared with the bagged, non-chilled plants significant differences were noted ($p<.05$) both for dry weight and for dry weight/fresh weight ratio. This was true even though the chilled plants did not appear to have necrotic leaf tissue. When the effects on the bagged, chilled plants are compared with the effects on the non-bagged, chilled plants it is apparent that bagging did have a protective effect. Thus, water stress is involved in chilling injury in maize, but the effects of chilling cannot be entirely eliminated by protection from water stress. The occurrance of water stress would itself require explanation. Although stomatal opening rather than

Table 16. Dry Weight (DW) and Dry Weight/Fresh Weight (DW/FW) (V3xB14)-2-1.

| Treatment | DW | DW/FW |
|---|---|---|
| Non-Chilled, Bagged 2 Days | .267(.017) a [1] | .078(.003) a [1] |
| Chilled, Bagged 2 Days | .218(.017) b | .084(.003) b |
| Chilled, Not Bagged | .128(.031) [2] | .122(.010) [3] |

[1] Values for DW and for DW/FW not having a letter in common are significantly different ($p<.05$) in a Duncan's multiple range test. Values in parentheses are standard deviations.

[2] This value was taken from Table 10.

[3] This value was taken from Table 11.

closing was noted following chillng of Phaseolus vulgaris (143), the mechanism for this response was not clear. Chilling of membranes has led to leakage so one might expect guard cells to lose water and close rather than open at low temperature. Perhaps guard cells are exposed to the leaked contents of surrounding cells, or perhaps stomatal opening is the result of extensive internal structural damage to the leaf, preventing proper mechanical action by the guard cells.

The protection offered by increased humidity suggests that damage in the field could be very severe following chilling. The coldest nights are usually dry and would normally be followed by days of low humidity and high light intensity. If sunlight or wind quickly evaporated any formation of dew, damaged leaves would rapidly become dehydrated.

When plants were chilled in plastic bags and allowed to remain in them for two days following chilling, chlorotic bands were present, being especially pronounced on the second leaves, and appeared where necrotic areas developed in the previous experiment where plastic bags were not used. These "Faris bands" have been observed in C4 grasses following exposure to low temperature. Slack, et al. (124) suggested that abnormal plastid development in cells of the band area may be due to failure of plastid ribosome production.

The results of these experiments indicate that although there were large genotypic differences in response to chilling at 2-3C much of these differences appeared to be due to the size of the plant at the time of chilling. There were some differences that could not be attributed to plant size. A comparison of the susceptibility of hybrids

and of their parents did not reveal clear inheritance patterns for susceptibility to chilling. Maintaining the relative humidity at 100% during and subsequent to chilling had a protective effect, but did not entirely eliminate chilling injury. Thus water stress is not the only factor involved in chilling injury to maize leaves. Considering that chilling reduced growth of some of the genotypes as much as 50% in the week following chilling (Chapter IV), this type of chilling injury deserves further study. A more effective means of differentiating genotypes is needed. Perhaps primitive maizes or teosintes could be found which would exhibit large degrees of resistance regardless of size of the plants at the time of chilling.

# C H A P T E R  VI

# FATTY ACID COMPOSITION OF POLAR LIPIDS OF MAIZE SHOOTS

## Introduction

Several researchers regard membrane lipids as the primary sites of chilling injury (68, 71, 114, 117). Membranes are thought to undergo changes at chilling temperatures. Phase changes of lipids from the normal liquid-crystalline state to the abnormal solid-gel state may occur. Phase separation may also occur, in which less fluid lipids separate from the more fluid lipids such that some areas of the membrane remain in a liquid-crystalline state while other areas assume a solid-gel state as temperature is lowered into the chilling range. Lipid phase changes have been suggested as the causes of changes in membrane permeability (92), respiration (70), and enzyme activities (107). There is some evidence that lipids from plant membranes do change phase at low temperatures (115, 140). One of the factors that determines the temperature at which lipids undergo phase transition is the relative content of unsaturated fatty acids. Increasing the degree of unsaturation of _in vitro_ lipids decreases the temperature at which phase change occurs (69). In the present experiments fatty acid composition of polar lipids of shoots was measured for several maize genotypes which differed in responses to chilling temperatures (Chapters II, III, IV, and V; 88). Fatty acid composition of the species _Zea diploperennis_ was also measured. Polar lipids of leaves are chiefly

galactolipids and phospholipids (146) and are thought to be the main constituents of the lipid fraction of membranes.

## Material and Methods

Plant Material. The maize inbreds A502, B73, Mp305, and (V3xB13)-2-1, the hybrid B73xMp305, and the species Zea diploperennis were used. Seed was obtained from sources as specified in Chapter II.

Growth Conditions. Light intensity, photoperiod, and nutrient conditions were as specified in Chapter II for the plants grown under light. Etiolated plants were grown in an unlighted incubator. The plants were grown 10-12 days at 22C in the light, 25-30 days at 16C in the light, or for 18-20 days at 16C in the dark prior to lipid extraction. Plants grown under light were exposed to a photoperiod of 14 hours light and 10 hours of darkness.

Fatty Acid Extraction and Measurement. Lipids from whole shoots were extracted by a method similar to that used by Folch, et al. (40). Shoots were steam heated for two minutes to inactivate lipases. 1.5 grams of shoots were mixed in a Virtis blender at 1/2 speed for 15 seconds with 10 ml of chloroform:methanol (2:1, v/v) and 100 mg/l of butylated hydroxytoluene (BHT), an anti-oxidant used to protect the double bonds of unsaturated fatty acids from oxidation. The homogenate was passed through a fine wire mesh strainer which served both to retain fibrous material and most of the aqueous fraction of the homogenate. The chloroform:methanol fraction containing the lipids, and a small amount of water was dried at 60C under nitrogen. Fatty acids of polar

lipids were esterfied by a method similar to Metcalf, et al. (82). The dried lipids were solubilized with 2 ml of methanol containing 0.5 N NaOH by heating for 5 minutes at 60C. Two ml of methanol containing 14% boron trifloride were added and the solution was heated for an additional 5 minutes at 60C. The lipid solution, 1 ml of saturated aqueous solution of NaCl, and 5 ml of petroleum ether containing 100 mg/l of BHT were put in a separatory funnel and shaken for one minute. The aqueous phase was discarded and the ether phase containing the esterified fatty acids was dried at 60C under nitrogen. One ml of cyclohexane containing 100 mg/l of BHT was used to dissolve the esterified fatty acids. Three ul of the exterified fatty acids were injected into a Varian Aerograph 600-D liquid-gas chromatograph with a column temperature of 200C and an injection temperature of 230C. The stainless steel column contained 10% diethylene glycol succinate. The dimensions of the column were 1.6 m by 3.13 mm (1/8") O.D. Peaks were compared with standard methyl esters (Sigma). Peak height x retention time was used in computing the relative percent of each fatty acid (47). The fatty acids 16:0, 16:1, 18:0, 18:1, 18:2, 18:3, and 22:0 made up more than 99% of the total fatty acids so other fatty acid components were not measured. Pigments were present in the samples that were grown in the light, but peaks for these occurred after the fatty acid peaks and did not interfere with fatty acid analysis.

Experimental Design. There were 8-9 replications within each treatment for each genotype. Analyses of variance were performed for double bond index (DBI), which is calculated as follows:

DBI = [% 18:1] + 2x[% 18:2] + 3x[% 18:3] (72). A separate analysis of variance was performed for plants grown at 22C; for plants grown at 16C in the light; and for plants grown at 16C in the dark.

## Results and Discussion

Fatty acid content of plants grown at 22C in the light is presented in Table 17. The F-value for DBI was 2.78, short of the value required for significance ($p<.05$). In all of the inbreds the predominant saturated fatty acid was palmitic acid (16:0) and the predominant unsaturated fatty acid was linolenic acid (18:3). There was a tendency for the inbreds B73 and (V3xB14)-2-1 to be higher in linolenic acid and lower in palmitic acid than the other two inbreds, but this did not result in a significant effect on DBI.

Although no significant differences were found in DBI of total polar lipids, the data do not rule out the possiblity that there were other important but undetected differences in fatty acid content. An analysis of total polar lipids of mitochondrial or chloroplast membranes may have revealed differences between genotypes, or perhaps an analysis of total phospholipids or of phospholipids from mitochondria or chloroplasts would have revealed significant differences.

Lowering the growth temperature might lead to differences in fatty acid composition between genotypes if some genotypes are beter able to adapt to the lower growth temperature. Fatty acid composition of plants grown at 16C was measured for plants grown in the light (Table 18).

Table 17. Fatty Acid Composition (%) and Double Bond Index (DBI) of Polar Lipids of Shoots Grown at 22C.

| Genotype | 16:0 | 16:1 | 18:0 | 18:1 | 18:2 | 18:3 | 22:0 | DBI |
|---|---|---|---|---|---|---|---|---|
| A502 | 13.9 (.5) | 3.4 (.7) | 2.4 (.4) | 3.5 (.4) | 18.1 (1.6) | 57.5 (1.4) | 1.2 (.2) | 2.16 [1] (.07) |
| B73 | 13.5 (1.1) | 3.1 (.4) | 2.4 (.5) | 3.2 (.4) | 18.7 (2.5) | 57.9 (2.2) | 1.2 (.3) | 2.18 (.09) |
| Mp305 | 14.6 (.6) | 3.8 (.7) | 2.5 (.3) | 3.5 (.3) | 18.4 (1.2) | 56.0 (1.2) | 1.3 (.4) | 2.12 (.08) |
| (V3xB14)-2-1 | 13.2 (.8) | 3.3 (.3) | 2.7 (.3) | 3.2 (.5) | 18.1 (1.1) | 58.4 (2.3) | 1.1 (.3) | 2.18 (.10) |

[1] There were no significant differences ($p<.05$) between genotypes in DBI. Values in parentheses are standard deviations.

Table 18. Fatty Acid Composition (%) and Double Bond Index (DBI) of Polar Lipids of Shoots Grown at 16C under Light.

| Genotype | 16:0 | 16:1 | 18:0 | 18:1 | 18:2 | 18:3 | 22:0 | DBI |
|---|---|---|---|---|---|---|---|---|
| A502 | 14.6 (.3) | 2.9 (.2) | 3.7 (.4) | 3.7 (.3) | 24.0 (.9) | 49.3 (.7) | 1.6 (.3) | 2.03 b[1] (.08) |
| B73 | 14.6 (.4) | 2.8 (.3) | 2.9 (.2) | 3.1 (.9) | 18.7 (.7) | 56.1 (1.0) | 2.1 (.2) | 2.12 c (.08) |
| Mp305 | 19.5 (.6) | 1.6 (.4) | 4.2 (.2) | 4.6 (.5) | 25.2 (.4) | 44.0 (.9) | 1.6 (.4) | 1.89 a (.09) |
| (V3xB14)-2-1 | 17.5 (1.2) | 3.0 (.1) | 3.0 (.6) | 3.9 (.5) | 27.6 (.7) | 42.1 (1.4) | 1.7 (.4) | 1.89 a (.09) |
| B73xMp305 | 12.5 (1.1) | 2.8 (.2) | 2.1 (.3) | 2.9 (.3) | 17.7 (1.0) | 60.4 (1.7) | 1.6 (.2) | 2.22 d (.08) |

[1] Values of DBI not sharing the same letter are significantly different ($p<.05$) in a Duncan's multiple range test. Values in parentheses are standard deviations.

Even though there were large differences in DBI's, the differences did not correlate with ability to grow at this temperature. The inbred (V3xB14)-2-1 had the smallest DBI, indicating that it had the least unsaturated fatty acids, but this was the fastest growing inbred at this temperature (Table 3). The hybrid B73xMp305 was also a fast growing genotype at this temperature but had the highest DBI. However, the DBI was correlated with chlorophyll content (Table 5), the genotypes with the most chlorophyll having the the highest DBI. These results are in agreement with a previous study showing a positive relationship between linolenic acid content and chlorophyll content in greening cucumber cotyledons (93). The reason for the close correspondence between chlorophyll content and linolenic acid is not yet known. When maize is grown under low temperature, linolenic acid synthesis may be blocked, which in turn may result in reduced chlorophyll synthesis; or chlorophyll syntheis may be blocked, resulting in reduced linolenic acid synthesis; or the synthesis of both molecules may be limited coincidentally by some other process.

Because of the close relationship between chlorophyll content and degree of fatty acid unsaturation, DBI was measured for etiolated plants grown at 16C in the dark. The results are presented in Table 19. Differences in fatty acid content were much smaller than for plants grown under light at 16C, but there were still significant differences. The plant with the most chlorophyll at low temperature was Zea diploperennis (Table 6). It also had the highest DBI. The plant with the least chlorophyll, (V3xB14)-2-1, had the lowest DBI. The growth

Table 19. Fatty Acid Composition (%) and Double Bond Index (DBI) of Polar Lipids of Shoots Grown at 16C in the Dark.

| Genotype | 16:0 | 16:1 | 18:0 | 18:1 | 18:2 | 18:3 | 22:0 | DBI |
|---|---|---|---|---|---|---|---|---|
| A502 | 20.9 (.3) | 5.8 (.6) | 5.1 (.3) | 8.1 (.2) | 46.4 (1.9) | 12.7 (.5) | 1.1 (.3) | 1.45 abc[1] (.06) |
| B73 | 20.2 (.1) | 5.5 (.1) | 5.0 (.3) | 8.5 (.1) | 46.4 (.1) | 12.9 (.3) | 1.5 (.3) | 1.46 ab (.05) |
| Mp305 | 21.7 (1.2) | 6.2 (.6) | 5.3 (.8) | 7.4 (.3) | 46.1 (1.8) | 11.2 (.4) | 1.3 (.1) | 1.40 cd (.06) |
| (V3xB14) -2-1 | 21.1 (.1) | 6.6 (.4) | 5.8 (.6) | 9.0 (.6) | 43.7 (.5) | 11.7 (.7) | 1.4 (.4) | 1.38 d (.07) |
| B73xMp305 | 20.8 (.2) | 5.4 (.4) | 4.7 (.3) | 8.6 (.6) | 46.8 (.9) | 12.3 (.2) | 1.5 (.2) | 1.41 bcd (.07) |
| Z.d.[2] | 20.9 (.9) | 6.0 (.6) | 5.2 (.6) | 9.1 (.2) | 38.0 (1.3) | 19.5 (.9) | 1.2 (.3) | 1.50 a (.08) |

[1] Values of DBI not sharing the same letter are significantly different ($p<.05$) in a Duncan's multiple range test. Values in parentheses are standard deviations.

[2] Zea diploperennis.

cabinet was opened once for watering, perhaps admitting enough light to trigger the synthesis of a small amount of chlorophyll in some genotypes. Zea diploperennis did appear to be slightly pigmented.

The data indicate that fatty acid composition of total polar lipids extracted from shoots would not be a useful criterion for selection of chilling tolerant lines of maize.

# C H A P T E R  VII

# RESPIRATION RATES AT 10C, 16C, AND 22C OF PLANTS GROWN AT 22C AND 16C

## Introduction

At chilling temperatures respiration rates of mitochondria have been found to be more reduced in chilling sensitive plants than in chilling resistant plants. An Arrhenius plot of respiration rate *vs* temperature showed a break at 12C for sweet potato, tomato, and cucumber, but there was no break for beets, potatoes, or cauliflower (70). The decreased respiration rate below 12C was thought to be due to membrane lipid phase changes which caused a corresponding disruption of enzyme activities in the mitochondrial membranes. If respiration in isolated mitochondria is affected by a temperature-induced phase change of lipids, then one would expect that *in vivo* respiration should also be affected. The present experiments measured dark respiration rates of the shoots of several maize genotypes and of the species *Zea diploperennis*.

## Materials and Methods

*Plant Material.* Four inbred lines of maize A502, B73, Mp305, and (V3xB14)-2-1, one hybrid, B73xMp305, and *Zea diploperennis* were used. Sources of plant material were as specified in Chapter II.

*Growth Conditions.* Plants were grown at either 22C for 10 days or at 16C for 16-20 days. Light intensity, photoperiod, and nutrient conditions were as specified in Chapter III.

Respiration. Respiration was measured as evolution of carbon dioxide from whole shoots. Carbon dioxide was measured by a LIRA Infra-Red Analyzer calibrated for 350-450 ppm of carbon dioxide, and was expressed as ml carbon dioxide per dry weight per hour. Measurements were taken at 22C, 16C, and 10C. There were 6-8 replications per genotype at each temperature.

## Results and Discussion

Respiration of plants grown at 22C is presented in Table 20. There were no significant differences between maize genotypes at any temperature. However, respiration of Zea diploperennis was significantly lower than maize at both 16C and 22C, but was similar to maize at 10C. Thus it appears that the chilling temperature of 10C had relatively less effect on the respiratory apparatus of Zea diploperennis than on maize. This result is in contrast to a previous experiment showing that growth of Zea diploperennis was reduced by chilling treatments as much as was the growth of maize (Table 9). However, the experiment presented in Table 9 did not actually measure growth rate at 10-11C but measured growth that occurred both during a week at 10-11C and one additional week at 22C. Perhaps growth of Zea diploperennis during the final week at 22C was adversely affected by exposure to one week at 10-11C. Comparisons of growth rate between Zea diploperennis and maize are also complicated by the fact that seed reserve of Zea

Table 20. Respiration (ml carbon dioxide per gr fresh weight per hour) at 3 Temperatures for Shoots Grown at 22C.

| Genotype | 10C | 16C | 22C | 10/22 [1] |
|---|---|---|---|---|
| Z.d. [2] | .14(.01) a[3] | .22(.03) a | .42(.02) a | .33 |
| B73xMp305 | .14(.01) a | .31(.03) b | .56(.04) b | .25 |
| A502 | .12(.02) a | .28(.02) b | .53(.04) b | .23 |
| B73 | .14(.02) a | .27(.03) b | .54(.07) b | .25 |
| Mp305 | .14(.02) a | .26(.04) b | .57(.05) b | .24 |
| (V3xB14)-2-1 | .12(.02) a | .24(.03) b | .53(.05) b | .22 |

[1] The ratio 10/22 is obtained by dividing respiration at 10C by respiration at 22C.

[2] Zea diploperennis.

[3] Values in each columnm that do not have a letter in common are significantly different ($p<.05$) in a Duncan's multiple range test. Separate statistical analyses were performed for respiration at each temperature. Values in parentheses are standard deviations.

diploperennis is much smaller. An analysis of dry weight production of etiolated shoots of maize and of maize x Zea diploperennis hybrids, which produce a seed closer in size to maize, might lead to a better understanding of the relationships between seed size, plant growth, and respiration.

Additional evidence that respiration at low temperature was not closely correlated to plant growth at low temperatures is indicated by a comparison of two maize inbreds. There were no differences in respiration rate of B73 and (V3xB14)-2-1 at any of the three temperatures but (V3xB14)-2-1 grew twice as fast as B73 at 16C (Table 4). These differences in growth rate can not be accounted for by a difference in photosynthetic rates because the faster growing inbred, (V3xB14)-2-1, was practically devoid of chlorophyll at 16C (Table 5). There may be several explanations for the lack of correspondence between dark respiration and growth rate at 16C. Perhaps respiration is uncoupled to various extents depending upon the genotype, so that some genotypes are less efficient in producing shoot dry matter from seed reserves. A partial uncoupling of oxidative phosphorylation was observed in maize following a much lower temperature, 0.3C (26). Another explanation for the lack of correspondence between dark respiration and growth at 16C might be that there were genotypic differences in the mobilization of seed reserves. The seed not only provides substrates for respiration but also provides the raw materials for structural molecules such as cellulose, proteins, and lipids. A final explanation may be that respiration and growth rate are actually

closely related but that the experimental design did not allow for detection of differences in respiration rate. Growth rate in Table 4 measured cumulative dry weight production during the first two weeks, but respiration was measured at 10 days for the plants grown at 22C. Perhaps seed reserve was limiting by the time respiration was measured. Respiration rates taken on younger seedlings might answer this question.

Dry weight production of (V3xB14)-2-1 was also greater than B73 at 22C (Table 3). If mobilization of seed reserve was the predominant factor in determining the difference in plant growth at this temperature then removal of the endosperm prior to germination might be expected to eliminate the difference in growth rate. When this experiment was done, there was no longer a significant difference in shoot dry weight after 10 days of growth: mean weight of 15 shoots of (V3xB14)-2-1 was .0078(.0020) and mean weight of 18 shoots of B73 was .0077(.0021) grams. The lack of significant difference when plants were grown without endosperm suggests that mobilization of seed reserve was important in determining growth rate in those plants grown with endosperm (Table 3). However, the large standard deviations prevent one from placing too much confidence in the validity of the results. The large standard deviations may have been due to variable damage to the embryos.

Respiration of plants grown at 16C was also measured, with the idea that there might be different abilities of the genotypes to adapt to the lower temperature. For maize inbreds, respiration rates measured at 22C were generally lower than for plants grown at the warmer temperature (Table 21). The two inbreds A502 and Mp305 had much lower respiration

Table 21. Respiration (ml carbon dioxide per gram dry weight per hour) at 3 Temperatures for Shoots Grown at 16C.

| Genotype | 10C | 16C | 22C |
|---|---|---|---|
| Zea diploperennis | .14(.02) a[1] | .27(.01) b | .48(.05) a |
| B73xMp305 | .14(.03) a | .33(.02) a | .53(.03) a |
| B73 | .13(.01) a | .23(.02) c | .42(.02) b |
| (V3xB14) -2-1 | .13(.02) a | .23(.02) c | .42(.03) b |
| A502 | .10(.01) b | .20(.02) d | .34(.05) c |
| Mp305 | .06(.02) c | .15(.03) e | .27(.05) d |

[1] Values in each column that do not have a letter in common are significantly different (p.05) in a Duncan's multiple range test. Separate statistical analyses were performed for respiration at each temperature. Values in parentheses are standard deviations.

rates at all temperatures than did plants grown at the warmer temperature. These were also the slowest growing inbreds at 16C (Table 3, Table 4). It appears that instead of adaptation to the lower temperature there may have been chilling injury to the respiratory apparatus by prolonged exposure to 16C. It is possible that chilling injury to the respiratory apparatus may have been the cause of slow growth at 16C. The fact that only prolonged exposure led to the differences between sensitive and resistant genotypes suggests that chilling temperatures involves more than simply causing phase transitions in mitochondrial membranes. One would expect that if phase transitions are involved in chilling injury, the transitions should be immediate upon lowering the temperature, and genotypic differences should be apparent. Phase separation of membrane lipids might require prolonged exposure to chilling temperatures and thus might offer an explanation of why the effects of chilling were not immediately apparent on respiration. Perhaps another explanation is that the synthesis of mitochondria or of enzymes or other components in mitochondrial membranes are affected by prolonged exposure to low temperature. On the other hand, it is also possible that respiration, as well as reduced growth, at low temperature may both be secondary effects of some other process.

Respiration rates of the hybrid B73xMp305 were not affected by the temperature at which the plants were grown, even though both parents had lower respiration rates when grown at 16C than when grown at 22C. A temperature-dependent heterosis was also apparent in this hybrid for

chlorophyl content at 16C (Table 5) and for growth at 16C (Table 4). The fact that respiration of the inbreds was affected by growth temperature but that respiration of the hybrids and of the heterozygous Zea diploperennis was independent of growth temperature suggests the possibility that inbreeding produces mitochondria that are more sensitive to chilling. An analysis of respiration vs temperature of mitochondria isolated from inbreds and hybrids might shed more light on the role of heterosis in the chilling sensitivity of mitochondria.

In summary, respiration of maize genotypes grown at 22C did not differ when measured at 10C, 16C, or 22C. Respiration of Zea diploperennis was lower at 16C and 22C than respiration of maize genotypes, but was similar to the maize genotypes at 10C. When plants were grown at 16C, respiration of A502 and Mp305 was generally lower at any temperature. These two inbreds were also the slowest growing inbreds at 16C in a previous experiment (Table 3), and in a previous experiment involving cold field conditions (88). The occurrance of reduced respiration as a result growing plants at 16C, along with the data showing reduced chlorophyll content at 16C, show that chilling injury can occur as a result of prolonged exposure to this relatively mild chilling temperature.

# C H A P T E R  VIII

## SUMMARY AND CONCLUSIONS

One of the inbreds, Mp305, was quite sensitive to chilling during imbibition. This inbred also had a low emergence rate under cold field conditions (88), suggesting that the imbibitional chilling test may be an effective way to screen out genotypes that are sensitive to cold soils.

Germination following cold imbibition of two other inbreds, A502 and B73, was also greatly reduced. The sensitivity of B73 was in contrast to its relative resistance in a field test (88), possibly indicating that resistance to imbibitional chilling and ability to germinate in cold solils are separate traits.

The occurrance of a large maternal effect was observed for imbibitional chilling sensitivity of the reciprocal hybrids B73xMp305 and Mp305xB73. This was in contrast to a lack of maternal effects for chlorophyll content (Table 5) and for dry weight production at 16C (Table 3). Previous work (14) also reported maternal effects for imbibitional chilling sensitivity in maize. The chilling sensitive site may be the maternally inherited seed coat and/or the 3N endosperm, 2/3 of which is maternally inherited. Germination in cold soils may involve two separate genetic processes. The first process may determine survival following cold imbibition by dry seeds and would be determined by the maternal parent. The second process would be germination at low temperature of seeds which survived cold imbibition, and would be

determined by the genotype of the embryo. Simon (121) proposed that reduced survival following imbibitional chilling is due to solute leakage from the seed, and that the low temperature limit for germination is due to protein denaturation. The data of the present experiments do not provide information as to whether imbibitional chilling sensitivity and germination at low temperature are separate genetic traits.

Growth rate of maize seedlings at 16C for the first two weeks (Table 4) again showed that A502 and Mp305 were the most sensitive inbreds and that B73 and (V3xB14)-2-1 were the most tolerant. Growth rate at 16C and survival of imbibitional chilling both appear to be closely related to field performance of seedlings because in a field test for cold tolerance B73 and (V3xB14)-2-1 were the most tolerant and A502 and Mp305 were among the most sensitive (88). Chlorophyll content at 16C appears to have had no relationship to field performance. The most chlorotic inbred at 16C, (V3xB14)-2-1, was the second best inbred in the field test, as measured as a composite of percent emergence, dry weight at 30 days, and time to emergence. However, if at 30 days in the field seedlings of (V3xB14)-2-1 were still dependent on seed reserve, the effects of reduced chlorophyll might not have yet been detectable. Chlorophyll content may influence longer-term dry weight production under cold field conditions. Perhaps the field performance of (V3xB14)-2-1 would be even better if it were less sensitive to low temperature induced chlorosis.

The responses of warm-grown plants to chilling appear not to have been associated with chilling tolerance in the field. The two poorest inbreds under field conditions, A502 and Mp305, were less affected by chilling at 2-3C than were B73 and (V3xB14)-2-1. However, the influence of plant size seemed to be the over-riding factor in determining sensitivity to chilling, rather than genotype. Thus, differences in field performance would be extremely variable, depending on the size of plants during sudden cold spells. Considering the magnitude of growth reduction following the chilling treatment of 2-3C and the fact that growth reduction occurred after only 12 hours of chilling, it seems likely that all of the genotypes in these experiments could be chill-injured under cold field conditions. Thus it would seem worthwhile to 1) develop a procedure for differentiating genotypes which is not influenced by plant size, and 2) look for genotypes with a greater degree of resistance to sudden at any plant size.

A root temperature of 13-16C offered little protection to shoots that were chilled at 2C, indicating that shoots may be directly affected by the treatment. Maintaining a relative humidity of 100% reduced chilling injury to shoots but did not eliminate damage even with prolonged protection (during chilling and two days after chilling). Thus, injury does not appear to be simply a temperature-induced water stress effect.

Double bond index of fatty acids of polar lipids of plants grown at 22C did not differentiate among genotypes. Double bond index of shoots grown at 16C correlated with chlorophyll content, such that those

genotypes having more chlorophyll had higher DBI's. DBI was unrelated to ability of the maize genotypes to grow at low temperature. It is possible that some other parameter of unsaturation such as fatty acid composition of phospholipids might be more useful in differentiating between maize genotypes.

Differences in respiration rates of maize were seen when plants grown at 22C were compared to plants grown at 16C. A502 and Mp305, the two inbreds which had the slowest growth rates at 16C (Table 4) and performed most poorly in a field test (88), had reduced respiration rates when grown at 16C. Respiration rates measured at 10C, 16C, and 22C were not different among inbreds when plants were grown at 22C. These results suggest that if the respiratory apparatus is a primary site of chilling injury, the effects of chilling require prolonged exposure to the chilling temperature. This complements results of Sasson (119) that respiration in cucumber was not immediately affected by chilling as was electrolyte leakage. The results argue against the idea that phase transition in mitochondrial membranes is the primary mechanism for chilling injury because phase transitions of lipids should be nearly instantaneous as temperature falls below the transition temperature. Phase separation of lipids could be a time-dependent process and thus might be used as an explanation for the delayed effects of chilling on respiration.

Since Zea diploperennis was found growing at 2250 to 2400 m elevation in Mexico (55), it was expected that this species might exhibit greater chilling resistance than maize genotypes which are

adapted to warm growing seasons. Zea diploperennis, as well as the interspecies cross B73 x Zea diploperennis, proved to quite resistant in terms of chlorophyll content of plants grown at low temperature. However, in terms of growth at 16C and in terms of growth following chilling at either 10-11C or 2-3C, Zea diploperennis was not superior to maize. Because of the much smaller seed reserve, Zea diploperennis may become dependent on photosynthesis sooner than maize, which has a larger seed reserve. Chlorophyll development at low temperature may therefore be more important for young Zea diploperennis than for maize. The latitude and altitude at which Zea diploperennis was found growing indicates that it is subjected to high light intensity. Chlorosis has been found to occur when low temperature is coincident with high light intensity. Resistance to low temperature chlorosis would be a desirable adaptation for a plant such as Zea diploperennis with a small seed reserve growing at cool temperatures under high light intensities.

Temperature-dependent heteroses were found for three traits in the hybrid B73xMp305: 1) Chlorophyll content at 22C was similar to the parents at 22C but was much higher than the parents when plants were grown at 16C. 2) Respiration rates of plants grown at 22C were similar, but respiration rates of the hybrid were generally greater than rates of the parents when plants were grown at 16C. 3) Growth rate of the hybrid and its parents were similar at 22C, but the hybrid grew faster at 16C for 30 days than did the parents. The temperature-dependent heteroses for growth may simply have been due to greater chlorophyll content and greater photosynthesis at 16C. The occurrances of temperature-dependent

heteroses for chlorophyll content and maintainance of the respiratory capacity by cold-grown plants is not as easily explained. Langridge (62) produced a temperature-dependent heteroses in plants by inbreeding for 20 generations at 22C. There was no hybrid vigor at 22C, but when plants were grown at 31C the F1 plants grew normally and the inbred parents died. The explanation given was that over the 20 generations each parent had developed separate heat-sensitive mutation(s) which had become homozygous. The hybrid presumably contained one allele for each non-heat sensitive enzyme. A similar explanation may apply for maize inbreds. Inbreeding for many generations at non-chilling temperatures may lead to the homozygous condition for many enzymes which are either 1) cold sensitive themselves, or 2) produce products such as lipids that are cold sensitive. This explanation of heterosis is called the "dominance" theory because it presumes that there is one best allele for each gene.

The "marginal overdominance" theory of heterosis (141) might also explain temperature-dependent heteroses in maize. This theory postulates that different alleles are best adapted to different environments. An inbred maize line may have many enzymes which are represented by dual copies of alleles adapted to warmer temperatures, while the hybrids would more likely have one allele of each pair that is adapted to cooler temperatures.

Based on the present experiments and on previous studies of germination at low temperatures (45; 139), survival of imbibitional chilling, germination at low temperature, growth at low temperature,

chlorophyll conent at low temperature, and resistance to sudden chilling appear to be traits that are under genetic control in maize. Growth at low temperature and chlorophyll content were seen to be genetically independent in Chapter III. The low temperature limit for germination may depend on protein denaturation (121), while seedling growth at slightly higher temperatures may be controlled by different genes. The determination that response to sudden chilling is a separate genetically controlled trait(s) will depend on the development of improved experimental techniques for differentiating genotypes regardless of plant size. It is possible that growth processes at low temperatures may be dependent upon properties of mitochondrial membranes, that chlorophyll synthesis at low temperatures may be dependent on properties of the chloropolast membranes, and that response to sudden chilling may be predominantly determined by properties of the plasmalemma at low temperatures. Properties of these membranes at low temperatures could be under separate genetic control.

If experimental evidence provides further support for the idea that all of the above traits have some degree of genetic independence, the following sequence of selection procedures may produce more generally cold tolerant maize plants than has selection based on a single criterion such as low temperature germination (45) or early seedling growth rate (37):

1. Imbibitional chilling: Reduce moisture content of seeds to 5% and soak seeds at 2C for 48 hours.

2. Low temperature germination: Let the imbibitionally chilled seeds germinate at 10C, selecting those which germinate soonest.

3. Seedling growth and chlorophyll content: Grow the selections from above at 16C, selecting the largest and greenest plants after 2-3 weeks.

4. Sudden chilling: Subject the selections from above to chilling at 2C for 48 hours, grow the plants at 22C for one week, and select those plants with the least amount of necrotic tissue and/or the most growth following chilling. By choosing only large plants at 16C, the influence of plants size on susceptibility to chilling at 2C might be reduced.

5. Transplant the remaining selections to the field and select for other desirable characteristics.

The above sequence would necessarily have to be repeated for several generations in the development of new chilling resistant inbred lines. However, it is not necessarily true the chilling resistant inbreds would produce chilling resistant hybrids. The selection sequence could also be used to select for chilling tolerance among hybrids.

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